A new mechanism to confer resistance to *Alfalfa mosaic virus* infection in white clover and tobacco using mutations to the ATP binding motif.

A thesis submitted as a requirement for the degree of

Doctor of Philosophy

by

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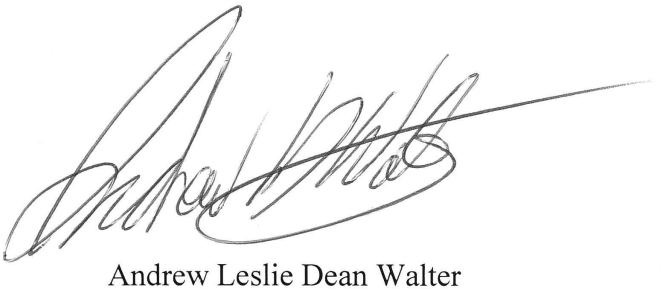
DECLARATION

I declare that the results presented in this thesis are my own work, except where otherwise stated, and were obtained under the supervision of Dr T.J. Higgins and Dr Paul Chu from CSIRO Plant Industry, and Dr Malcolm Whitecross from the School of Botany and Zoology at the Australian National University



Andrew Leslie Dean Walter

August 2001

FOREWORD

There is not one person more privileged than I to have had the support of so many fantastic and exceptional people and organisations in undertaking my PhD. It has been the opportunity, challenge and experience of a lifetime all in one. It is not normal for a long foreword to be included in a PhD thesis, but I think I can say, few elements of my PhD have been normal.

My PhD would not have been possible without having the best supervisors on earth – T.J. Higgins, Paul Chu and Malcolm Whitecross. Always committed and enthusiastic about my research work and extra-curricular activities, there is no way I could have done what I have in the lab or outside it without your support. How can I ever thank you enough for your wisdom, encouragement, advice and help throughout what has been an ambitious project. To have got me to the end via the 'route' taken I think is a reflection on you all. "Mission impossible accomplished and landed!!". I hope you enjoyed the 'flight' as much as I have – what a fantastic learning experience – and that it has ultimately all been worthwhile.

T.J, I hope that some part of your professional and personal qualities have been imparted to me (except for the 'give the PhD student a hard time' qualities), especially your sincerity and honesty. If I am lucky enough that they have in some small way, I hope I am able to make great use of such in my future work and personal activities. At all times I have thoroughly enjoyed being your student. I hope the 'flight' was not too rough or too long – but I learnt so much and I hope you enjoyed it as much as I did. You are a true champion and great friend.

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your commercial crops on the farm one day soon!! You are an inspiration that will always be remembered.

Malcolm, how you ever had the patience for me I will never know. You always know you have a great friend when you can go for so long without contact and then be able to talk about anything when contact is made. What a fantastic career you have had and what a privilege it has been to be the last of your 34 graduate students! I hope it has not been a case of the last being the worst, but I suspect it is. There is no way I would have stuck at the project without your support and encouragement as a third party and 'experienced and wise mind' to what seemed on many occasions impossible difficulties and circumstances to be overcome.

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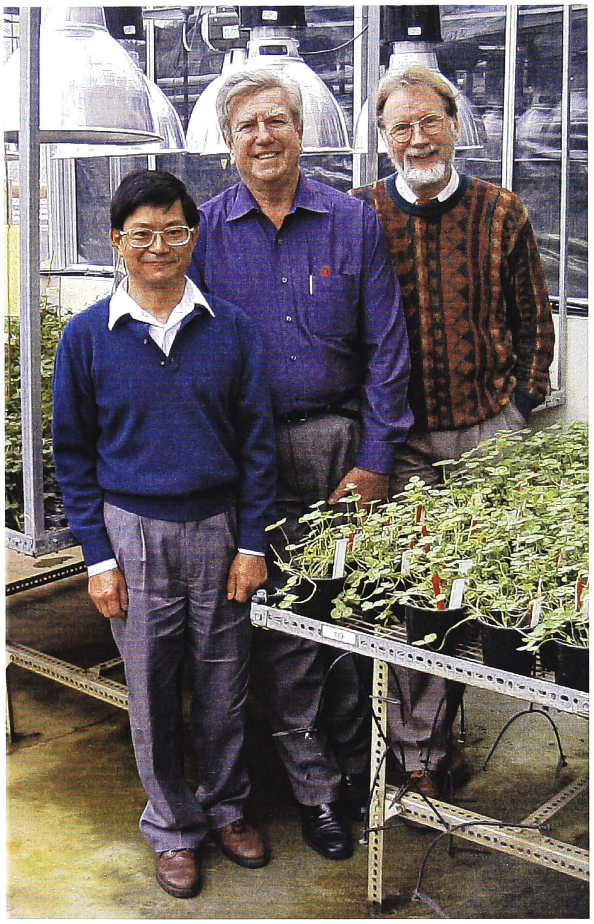
To my many great friends too numerous to individually acknowledge, a big thanks. Nigel and Kathryn, Ben and Fiona, Yasmina, Michelle, Anthony, Jim, thanks heaps.

How would I have ever known what doing a PhD could possibly involve inside and outside the lab. To have had the experience of high level academia, government, industry, science and politics I am in my mind, the luckiest person on earth.

THE BEST SUPERVISORS



l to r: Dr Paul Chu, Dr Malcolm Whitecross and Dr 'TJ' Higgins

Thank you for your unreserved support, dedication and commitment

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I would like to acknowledge the generosity of Professor John Bol from the Institute of Molecular Plant Sciences, Leiden University, The Netherlands, for making available to me the Alfalfa Mosaic Virus genomic infectious clones (AMV RNA 1-4 genomic clones, respectively called, pCa17T, pCa27T, pCa32T and pCa42T).

I would like to thank Jenny Gibson for her advice and assistance with the transformation and tissue culture of the white clover, Nick Hagan for his advice and assistance with carrying out the RT-PCR reactions and Dr John Watson for his much appreciated and dedicated assistance with the northern blots.

All statistical tests were undertaken by Warren Muller, CSIRO Mathematical and Information Sciences.

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ABSTRACT

A qualified claim is made that a new strategy to confer resistance to virus infection has been identified by transforming plants with a viral replicase gene in which the highly conserved ATP binding motif is mutated. The putative ATP binding motif (P-loop) in the RNA 1 gene of the *Alfalfa mosaic virus* (AMV) was found by mutation analysis to have an essential role in virus infection.

Transgenic plants generated from single independent transformation events containing a AMV RNA 1 gene construct with the 35S promoter and nos terminator with mutated P-loop motifs showed a range of resistant phenotypes and attenuation of the symptoms of AMV infection. In contrast, plants containing the wild type form of the AMV RNA 1 gene showed no resistant phenotypes or attenuation of virus infection symptoms. By visual and ELISA analysis the plants transformed with the wild type gene had the same susceptibility to virus infection as untransformed control plants. These results have been demonstrated in both tobacco (*Nicotiana tabacum* cv. W38) as a model system and white clover (*Trifolium repens* cv. Haifa) as a commercial plant species with high susceptibility to infection by AMV.

Whilst northern blot and RT-PCR analysis was unable to conclusively detect transgene message, the attenuation of virus infection only in plants containing the modified form of the AMV RNA 1 gene suggests that it is a protein-mediated form of resistance to virus infection.

ABBREVIATIONS

35S	35S RNA promoter of *Cauliflower mosaic virus*
AMV	*Alfalfa mosaic virus*
ATP	adenosine triphosphate
cDNA	copy DNA
cp	coat protein
dH_2O	deionized sterile water
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
ds	double stranded
E.coli	*Escherichia coli*
EDTA	ethylenediaminotetra-acetic acid
ELISA	Enzyme linked immuno-sorbent assay
GTE	glycerol tris EDTA
nos	transcriptional terminator of the nopaline synthase gene
*npt*II	neomycin phosphotransferase II
PCR	polymerase chain reaction
PEG	polyethylene glycol
RNA	ribonucleic acid
RNase A	ribonuclease A
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl phosphate
ss	single stranded
SSC	Sodium chloride sodium citrate buffer
tobacco	*Nicotiana tabacum*
white clover	*Trifolium repens*

TABLE OF CONTENTS

CHAPTER 1 – LITERATURE REVIEW.

1. INTRODUCTION.

White clover is a critically important pasture plant in Australia, particularly to the Dairy Industry. This perennial pasture legume is regarded as the 'pasture engine' of high rainfall and irrigated pastures – as a productive source of protein and highly digestible biomass and of fixed atmospheric nitrogen. Viruses are serious pathogens of plants. Studies of, and research on, plant viruses have been responsible for significant advances in our knowledge of their gene structure and function. The substantial advances in biochemistry and biotechnology in the past two decades has further revealed the significance and diversity of plant viruses. Although arguably as diverse and as significant as bacterial and fungal pathogens of plants, viruses have attracted significant attention only with the advent of molecular biology. Plant virology and biotechnology now offers the opportunity to further the frontiers of biological knowledge and for significant gains to be achieved in commercial plant productivity.

Alfalfa mosaic virus (AMV) infects at least 599 species of plant and is transmitted via seed, pollen, aphids and mechanical inoculation (Edwardson and Christie, 1986). In white clover, AMV infection reduces fresh foliage weight by up to 60% (Garrett, 1990). White clover cultivars with resistance to AMV infection could potentially increase total pasture production, with grass species present, by 30% (Mason, 1993).

In the first part of this Chapter, a review of the literature introducing plant viruses and the characteristics of AMV as a virus and as a pathogen of white clover is given. In the second part, the literature is reviewed with respect to the breeding, by conventional or molecular biology techniques, of resistance to virus infection in plants. In the third part of this Chapter, a new approach to confer resistance to AMV in white clover is proposed – the focus of this thesis.

1.1. Classification of plant viruses.

The primary characteristic by which plant viruses are classified is on the basis of their genetic material, either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) and whether it is double stranded (ds) or single stranded (ss). If the nucleic acid is in the ss form then it can be either in the positive sense, where the nucleotide sequence is able to be read directly for translation or in the negative sense, that requiring a complementary copy to be synthesized before translation. Further divisions of plant virus classification can be made on the presence or absence of an envelope (an additional protective layer to the coat protein). The different groups of plant viruses and some examples of such are illustrated in Figure 1.

1.2. *Bromoviridae* family of plant viruses.

Of the 27 recognized groups of plant viruses with a single stranded RNA genome in the positive sense, four have a similar tripartite genome (Neeleman *et al*, 1993). These belong to the Family *Bromoviridae* with the five genera being *Alfamovirus*,

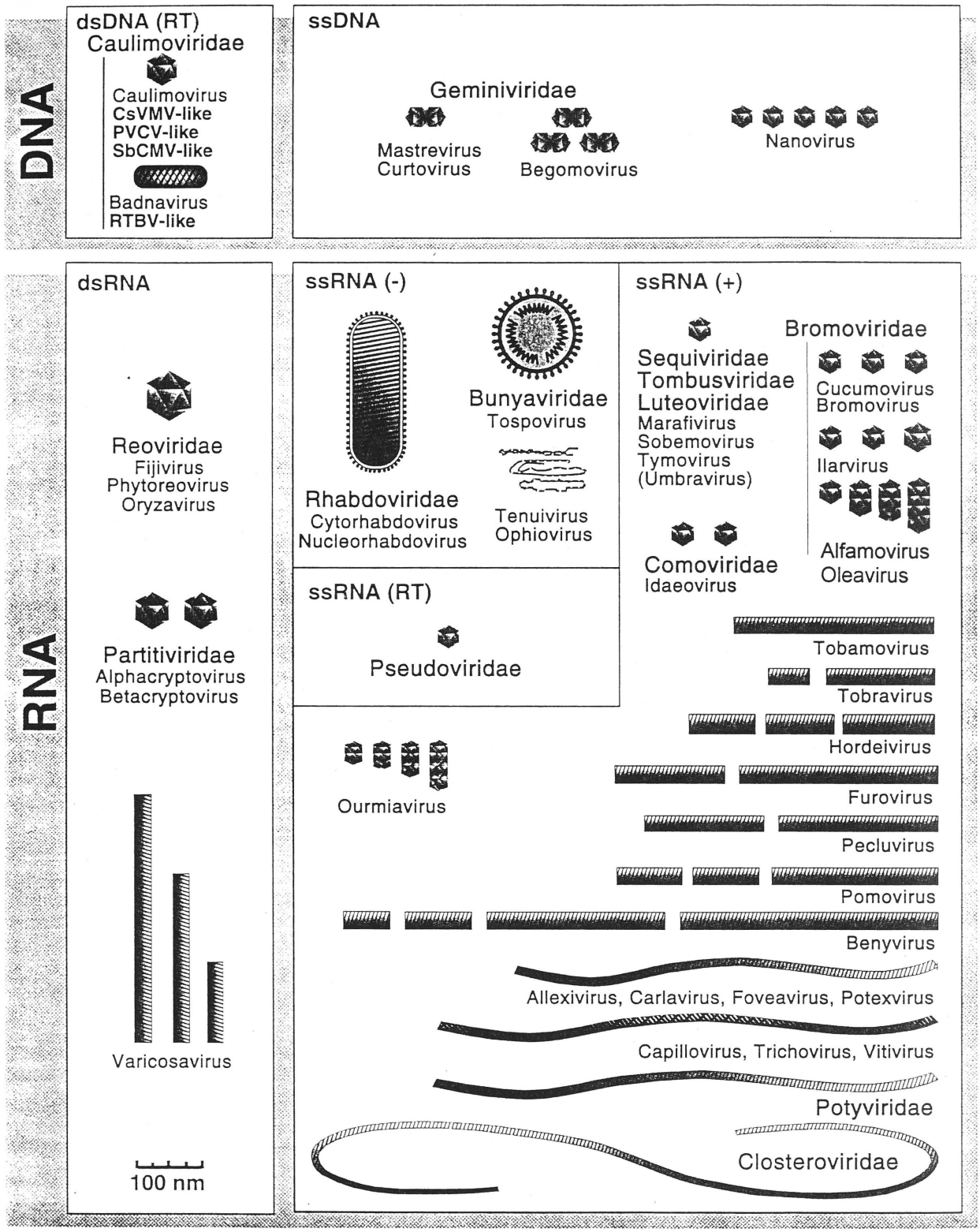


Figure 1: Families and Genera of viruses infecting plants.

(Adapted from van Regenmortel et al, 2000)

Bromovirus, *Cucumovirus*, *Ilarvirus* and *Oleavirus* (van Regenmortel *et al*, 2000). The inclusion of the genus *Oleavirus* into this family was only recently ratified by the International Committee on Virus Taxonomy (Pringle, 1998).

Viruses belonging to these genera have three genomic RNAs which are encapsulated by viral encoded coat protein synthesized via subgenomic RNAs. The RNA replication genes of these viruses, located in RNAs 1 and 2, have domains of significant sequence homology with RNA 1 having methyltransferase and helicase domains and RNA 2 having the 'GDD' polymerase motif (Haseloff *et al*, 1984; Ahlquist *et al*, 1985; Goldbach *et al*, 1991). The genome of each tripartite virus is identical in structure and similar in size. The genome organization and function of AMV is illustrated in Figure 2. The *Alfamovirus* and *Oleavirus* genera are unique in that they have only a single virus representative, *Alfalfa mosaic virus* and *Olive latent virus 2*, respectively.

There are some differences between viruses belonging to the five genera. Viruses belonging to the *Bromovirus* and *Cucumovirus* genera are infectious when a mixture of the genomic RNAs are inoculated onto susceptible plants. Distinctively, those of the *Ilarvirus* genus and AMV require a small amount of coat protein (CP) or subgenomic messenger RNA encoding the CP to be inoculated with the genomic RNAs for infection (van Vloten-Doting, 1975; Smit *et al*, 1981). In addition to its structural role, the CP of ilarviruses and AMV therefore has a function in infection which has been termed "genome activation" (Neeleman *et al*, 1993).

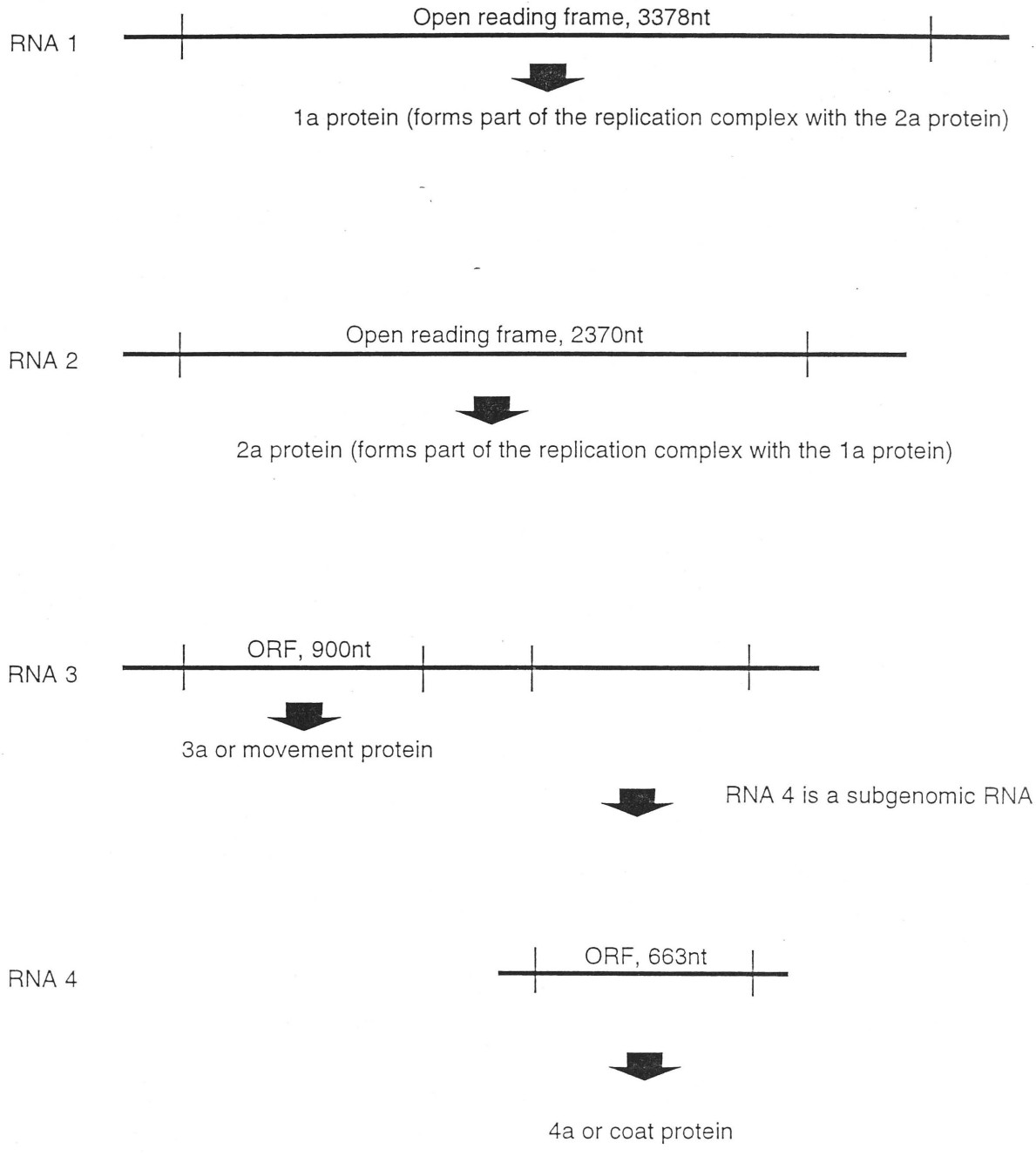


Figure 2: Genome organisation of tripartite viruses, such as Alfalfa Mosaic Virus. Alfalfa Mosaic Virus is monocistronic and codes for the 1a protein which has a putative methyltransferase and a putative helicase domain. RNA 2 is monocistronic and codes for the 2a protein which is often referred to as the polymerase gene. RNA 3 is bicistronic, encoding at the 5' end the 3a protein which is referred to as the movement protein. RNA 3 encodes the coat protein at the 3' end which is expressed via a subgenomic RNA called RNA 4.

The Bromoviridae viruses share the same genome organization and terminology.

(Diagram adapted from Matthews, 1991)

The 3'-termini of *Bromovirus* and *Cucumovirus* RNAs contain tRNA-like or stem loop structures which are not found in other tripartite viruses (Perret *et al*, 1989; Symons, 1979) but which have no particular affinity for their CP (Giege, 1996). AMV has high affinity binding sites for the CP at the 3'-termini of the genomic RNAs. The binding of the coat protein to the 3'-termini is thought to play a critical role in the replication of the virus genome (Smit *et al*, 1981; Nassuth and Bol, 1983; Houwing and Jaspars, 1986; Houwing and Jaspars, 1987). Transgenic plants expressing AMV RNAs 1 and 2, did not accumulate positive strand RNA until RNA 3 was inoculated onto the plants, thereby indicating that the coat protein plays a role in regulating the accumulation of positive strand viral RNA (Thole *et al*, 2001).

1.3. *Alfalfa mosaic virus* as a unique virus.

Whilst AMV has substantial nucleotide sequence similarity with other tripartite viruses, it is most closely related to the ilarviruses on morphological and biochemical features but it does have a number of distinguishing features (Sanchez-Navarro and Pallas, 1997; Shiel and Berger, 2000). A major difference between the genomes of AMV and the ilarviruses sequenced to date is the presence in the ilaviruses of a conserved open reading frame that overlaps the 3' end of the open reading frame of RNA 2 (Bol 1999). A subgenomic messenger corresponding to this open reading frame has been identified in infected plants (Xin *et al*, 1998). This subgenomic RNA is termed RNA4a (Bol 1999). Viruses belonging to the *Cucumovirus* also have a subgenomic message at a similar position in RNA 2 but this is smaller and has little similarity to those of the *Ilarvirus* genera (Ding *et al*, 1996).

AMV has bacilliform virion particles whilst those of the *Ilarvirus* genera have quasi-isometric or occasionally bacilliform particles (Matthews, 1991). The 3'-terminal regions of the genomic RNAs of *Tobacco streak virus* (TSV), an *Ilarvirus*, and AMV have no sequence homology. Atomic absorption analysis found TSV native virions to contain one zinc atom per four protein subunits and AMV to have one zinc atom per two protein subunits (Sehnke *et al*, 1989). *Prune dwarf virus*, an *Ilarvirus*, CP does not contain any of the zinc-finger motifs found in TSV and AMV CP (Bachman *et al*, 1994). TSV and AMV do have in common a sequence of stable hairpin structures flanked by the tetranucleotide sequence AUGC (Koper-Zwarthoff and Bol, 1979; Koper-Zwarthoff and Bol, 1980).

AMV also appears to be unique among the plant viruses in the variations exhibited in the types of inclusion bodies it induces in the cells of infected plants (Hull, 1970; Wilcoxson *et al*, 1974; 1975). The induction of raft-shaped aggregates of AMV particles in the cytoplasm is a diagnostic characteristic of AMV infection in plants (Edwardson and Christie, 1986).

Throughout the literature AMV is often regarded or classified as an *Ilarvirus* but the International Committee on Virus Taxonomy (ICTV) has AMV belonging to its own genus (van Regenmortel *et al*, 2000). This would seem well justified given the above described unique characteristics of the virus.

1.4. AMV as a significant plant pathogen.

AMV has been reported to infect 599 species of plants in 245 genera in 68 families (Edwardson and Christie, 1986), including *Trifolium ambiguum* that was otherwise thought to be resistant to viruses that infect pasture legumes (Wijkstra and Guy 1996). In the *Fabaceae*, AMV infects 156 species in 32 genera (Edwardson and Christie, 1986). Due to the wide host range of AMV it is possible for it to over winter or over summer in different species and then be transmitted by many different aphid species to new germinating or growing plants to allow it to persist in many natural and agricultural environments – this has been identified as being a particular problem in annual medics used in Australian agriculture (McKirdy and Jones, 1994).

In surveys of pastures containing different legumes, AMV is often found at a greater proportion of sites and at such sites it infects a higher proportion of plants than other viruses (Helms *et al*, 1993; Rahman and Peaden, 1993; Norton and Johnstone, 1998). Given the lack of resistance to AMV infection amongst pasture plants a major issue confronting farmers and commercial seed producers is the level (threshold) of AMV in seed which is acceptable so as to reduce the impact of the virus on production, although it is highly probable that infection will eventually occur (Jones 2000).

The wide host range combined with seed, pollen, aphid and mechanical transmission of the virus makes cultural methods of control almost impossible (Pathipanawat *et al*, 1995). AMV control in annual medics (*Medicago spp.*) can be achieved by the application of residual insecticides (Jones and Ferris, 1995) but this is not economic

or necessarily environmentally sustainable. AMV control in perennial legumes such as lucerne (*Medicago sativa*) and white clover (*Trifolium repens*) is only possible by the use of resistant cultivars.

1.5. AMV as a pathogen of White Clover (*Trifolium repens*).

In white clover, AMV reduces the leaf dry weight, stolon dry weight, stolon length, nodulation and the number of leaves per plant (Gibson *et al*, 1980, 1981). AMV infection in the Australian white clover cultivar, Haifa, reduced fresh foliage weight by 60% (Garrett, 1990). However, AMV infection of pasture plants such as lucerne (alfalfa) often gives rise to no obvious symptoms apart from a decrease in biomass production (Pathipanawat *et al*, 1995). AMV has been identified as being a major threat to the productivity of irrigated white clover pastures (McKirdy and Jones, 1995; McKirdy and Jones, 1997). In some pastures, up to 100% of white clover plants are infected with AMV (McKirdy and Jones, 1995) with the virus being found throughout the nation (Norton and Johnstone, 1998).

I am aware of only a single White Clover breeding line with enhanced, but not absolute, resistance to AMV infection, termed Southern Regional Virus Resistant (SRVR) (Gibson *et al*, 1989; Nelson and Campbell, 1993). Selected SRVR plants after cross breeding and selection show increased levels of resistance and the resistance trait was shown to be heritable although estimated to be at a relatively low level (Pederson and McLaughlin, 1994). In experimental plots with and without grasses, select SRVR white clover plants were shown to be superior to other

germplasm when challenged with AMV and other viruses (Taylor *et al*, 1995). Interspecific hybrids of *T. repens* and *T. ambiguum* show resistance to AMV infection although there is considerable difficulty in backcrossing such hybrids to white clover (Pederson and McLaughlin, 1989). A further complication in cross breeding resistance into white clover is that it is a tetraploid-like (disomic) self-incompatible species which requires at least 8 parental lines and in typical plant breeding circumstances around 20 parental lines need to be obtained for a new cultivar (Caradus and Woodfield, 1997). Given the work involved in breeding white clover it is important that the traits being introduced are effective and durable.

1.6. Importance of white clover and the impact of AMV resistant plants.

White clover is regarded as the most important pasture legume plant in high rainfall (>700mm per year.) and irrigated pastures in Australia. Such pastures are used by dairy farmers who produce milk with a farmgate value of more than of $AUS 2 billion dollars per year (Australian Dairy Industry, 1994 annual report). With processing and marketing adding many fold to the value of the farmgate product, the Australian Dairy industry and hence white clover, makes a significant contribution to the economy.

One of the primary limitations to the profitability of the Australian Dairy industry is the production and utilization of pasture (Mason, 1993). White clover as a productive legume has the potential to add a further $AUS 455 million per year to the farmgate value of the Dairy industry (Mason, 1993). Given that AMV is one of

the major pathogens of white clover, the commercial benefits of an AMV resistant cultivar are likely to amount to many hundreds of millions of dollars in the world, if not in Australia alone, through increased productivity and plantings.

A number of attempts have been undertaken to achieve AMV resistance by development of transgenic plants – such attempts are detailed in Section 2 of this thesis. There is a need for the development of alternative strategies which are free of intellectual property rights restrictions for the Australian industry and as a means to sustain resistance (to not select for resistance breaking strains of the virus) by using at least two or more unrelated mechanisms (eg. transgenes).

2. GENETIC ENGINEERING STRATEGIES TO CONFER VIRAL RESISTANCE IN PLANTS.

In a little more than the past decade there has been substantial research work in many laboratories around the world studying plant viruses and plant responses to viral inoculation and infection. The key impetus behind this work has been the potential commercial benefits of achieving virus resistant plants, and the gaining of greater knowledge of plant defence systems and the structure and function of different viral genomes.

In the first part of this period, sources of transgenes for plant virus resistance were solely divided into three broad categories; pathogen derived, plant derived and

thirdly non-plant and non-pathogen derived. Initially, it was anticipated that pathogen-derived transgene resistance would operate through expression of the protein. However, it was soon shown that untranslatable constructs, such as anti-sense constructs, were able to confer resistance. Furthermore, resistance was observed in plants expressing a pathogen derived transgene where there was no detectable mRNA or protein. The phenomenon where resistance is achieved in transgenic plants without a protein being expressed or detected is termed RNA mediated resistance or gene silencing induced resistance. In the past few years, great gains have been achieved in the understanding of how RNA mediated resistance mechanisms might work in plants.

In the remaining part of this section, an overview of research involving transgenes to confer resistance in plants – plant derived (Section 2.1), non-pathogen non-plant derived (Section 2.2), RNA mediated (Section 2.3) and pathogen derived (Section 2.4) – is presented. It is beyond the scope of this thesis to provide a full review of all examples relevant to each Section. However, special attention is given to those examples in each Section that involve AMV.

2.1 Plant derived transgenes for conferring resistance to viral infection.

Plants have defence mechanisms against pathogens, including viruses as demonstrated by resistance of some plants to different viruses and particular strains of viruses. The mechanisms of such 'natural' resistance are not known. Resistance to virus infection in plants has been shown to be controlled by single dominant

genes, incompletely dominant and recessive genes in the plant (Matthews, 1991). The hypersensitive response of plants is also thought to be involved in conferring resistance to viral infection (Goulden and Baulcombe, 1993).

The gene responsible for resistance to *Tobacco mosaic virus* in tobacco, termed the *N* gene, has been cloned and then expressed in transgenic tomato in which the resistance trait to *Tobacco mosaic virus* was expressed as in tobacco (Whitham *et al*, 1994; Whitham *et al*, 1996). To date this has been the only plant virus resistance gene cloned, despite some 19 being cloned for bacterial, fungal or nematode resistance (Cornelissen and Schram, 2000).

Three genes have been shown to be expressed in tissue involved in the hypersensitive response to pathogen infection. Genetically engineered tobacco plants expressing any one of these three pathogenesis-related (PR) proteins however failed to have any increase in resistance to viral infection (Linthorst *et al*, 1989).

A class of polypeptides called ribosome-inactivating proteins (RIPs) which have anti-viral properties have been identified in a number of plant species (Stirpe *et al*, 1992). Transgenic tobacco and potato plants expressing a RIP from pokeweed (*Phytolacca americana*) showed resistance to viral infection in proportion to the level of RIP. Transgenic plants expressing RIP were clearly abnormal, exhibiting a stunted and mottled phenotype (Lodge *et al*, 1993).

I am not aware of any plant derived transgenes which have been demonstrated to be effective against AMV.

2.2 Non-pathogen and non-plant derived transgenes for conferring resistance to viral infection

Plants expressing antibody genes specific to a virus have been shown to be resistant to infection by the virus for, *Artichoke mottled crinkle virus* (Tavladoraki *et al*, 1993), *Tobacco mosaic virus* (Voss *et al*, 1995) and by expressing the gene from a broad-spectrum antibody to both *Clover yellow vein virus* and *Potato virus Y* (Xiao *et al*, 2000). Transgenic plants expressing genes encoding ribonucleases have shown differing levels of resistance to viral infection (Watanabe *et al, 1995*; Mitra *et al*, 1996). Relative to other sources of transgenes, it appears that there has been little research undertaken into those that are non-pathogen and non-plant derived, which probably reflects the success of such in conferring resistance to virus infection.

2.3 RNA mediated viral resistance

Resistance to viral infection by transgenic plants expressing genes which are not translatable, antisense or where no transcript or protein can be detected has been observed on numerous occasions (reviewed; Baulcombe, 1996; van den Boogaart *et al*, 1998). Over time this has been called gene-silencing, homology dependent gene silencing and RNA mediated resistance. Indeed, much research now involves

the design and testing of gene constructs in plants to induce RNA mediated virus resistance.

Generally, when such resistance is observed, it is effective against high levels of inocula but only against pathogens containing a gene with sequence similarity to the gene being silenced – quite often it is strain or virus specific (Mueller *et al*, 1995; van den Boogaart *et al*, 1998). An observation also associated with RNA mediated resistance is transgenic plants recovering from viral infection a short period after inoculation (Goodwin *et al*, 1996) – the so called 'recovery phenomenon'.

RNA mediated viral resistance or gene silencing is associated with high levels of transgene expression in plants containing a number of copies of the transgene (Lindbo *et al*, 1993, Goodwin *et al*, 1996; Waterhouse *et al*, 1998). However, not always does a high copy number of a transgene necessarily give rise to gene silencing (Waterhouse *et al*, 1998). A model put forward to explain gene silencing was based on the idea that when sufficient quantity of RNA (either from a transgene or from a transgene and a virus) is present in a cell, RNA degradation enzymes are activated that are specific for the inducing, and hence, target RNA (Baulcombe, 1996). Such a model accounts for the high sequence similarity required in RNA mediated resistance, the capability to sustain resistance under high inocula concentrations and the 'recovery' phenomenon.

The presence of RNA fragments from the mRNA or virus in gene-silenced or virus resistant plants suggests that the degradation of the target RNA starts with

endonucleolytic cleavage at one or more sites and is then followed by exonucleolytic degradation (Lee *et al*, 1997; Metzlaff *et al*, 1997; Martienssen, 1998). Further analysis has found that both sense and antisense RNA fragments of around 25 nucleotides with sequence similarity to the target RNA are found in low amounts but consistently in plants showing RNA mediated viral resistance or gene silencing (Hamilton and Baulcombe, 1999; Mette *et al*, 2000; Llave *et al*, 2000; Dalmay *et al*, 2000; Hutvagner *et al*, 2000). This degradation is similar to the phenomenon observed in *Drosophila* called RNA interference (RNAi) in which ssRNA, of the sense or antisense orientation, when injected into embryos is not degraded but when homologous dsRNA is injected it is degraded within minutes and fragments of around 21 nucleotides are produced (Bernstein *et al*, 2001; Elbashir *et al*, 2001). It has been proposed that the apparent common system of gene inactivation be called post transcriptional gene silencing (PTGS) and that this system in plants is a form of defence against virus infection (Waterhouse *et al,* 2001).

Recently, targeting genes for silencing and virus resistance has been successful by the expression of so called 'hairpin RNA' constructs (Waterhouse *et al*, 1998; Wang *et al*, 2000). These gene constructs expressing an RNA that forms a hairpin like shape because it contains an inverted repeat sequence. The repeats are separated by a unique sequence which forms the loop for the hairpin. The 'hairpin RNA' constructs induce the PTGS system to degrade the target RNA (Waterhouse *et al*, 2000).

Whilst progress is being made to understand the mechanism(s) involved in RNA mediated resistance and PTGS, there still remain many aspects to be elucidated with

seemingly contradictory results yet to be reconciled. Nonetheless, whenever expression of transgenes is undertaken in plants, one needs to be conscious of the possibility, effects and symptoms of RNA mediated (PTGS) resistance.

2.4. Pathogen derived transgenes for conferring resistance to viral infection.

The idea that a pathogen's genome might itself be a source of resistance transgenes was based on the phenomenon of viral cross protection, in which plants infected with one strain were found to be immune from subsequent infection by another strain of the same virus (Sandford & Johnson, 1985). The pathogen derived transgenes used to date mostly involve the coat, replicase and movement protein genes from viruses.

Transgenic virus resistant plants were first produced in 1986 by genetic engineering tobacco plants to express the coat protein of *Tobacco mosaic virus* (Powell-Abel *et al*, 1986). The general applicability of this strategy has since been demonstrated for at least 20 different RNA viruses (Hull & Davies, 1992) and for up to three viruses in the one plant (Tricoli *et al*, 1995). The level of resistance achieved is often near complete immunity even at high levels of virus inocula.

Resistance to infection of AMV has been achieved by the expression of the entire AMV coat protein gene (Loesch-Fries *et al*, 1987; Tumer *et al*, 1987; van Dun *et al*, 1987; Anderson *et al*, 1989; Hill *et al*, 1991; Jayasena *et al* 1997; Xu *et al*, 1999; Jayasena *et al*, 2001; Timmerman-Vaughan *et al*, 2001; US Patent# 5,736,627) or mutants of the coat protein which do not have the C-terminal end (Yusibov and

Loesch-Fries, 1995). Resistance to infection was not achieved when a construct with a frame shift mutation in the AMV CP gene was expressed in tobacco (van Dun *et al*, 1988). Resistance was lower in plants expressing the AMV CP gene in the antisense form than in the wild type form (Jayasena *et al*, 1997). Transgenic tobacco plants expressing a form of the AMV CP in which an N-terminal serine residue had been changed to glycine were susceptible to infection by wild type virus but were resistant to a mutant form of the virus containing the same mutation (Taschner *et al*, 1994).

In the case of *Tobacco mosaic virus* (Powell-Abel *et al*, 1986), AMV (Loesch-Fries *et al*, 1987; van Dun *et al*, 1987; Hill *et al*, 1990; Yusibov and Loesch-Fries, 1995; Timmerman-Vaughan *et al*, 2001) and *Potato virus X* (Hemenway, 1988) the strength of resistance correlated positively with the levels of coat protein expressed in the transgenic plants. The mechanism of this resistance is referred to as protein mediated. In the other cases of CP mediated transgene resistance, the level of resistance correlated with the lack or low level of transcript and hence the lack of the CP. This is, as described in Section 2.3 of the Chapter, an example of RNA mediated resistance (Smith *et al*, 1994; Goodwin *et al*, 1996; English *et al*, 1996; reviewed by Finnegan and McElroy, 1994).

In general, coat protein mediated resistance is durable in the field and can give protection against closely related strains and viruses. Coat protein constructs expressed in plants can give protection against other strains and viruses where the proteins have a 60% or greater amino acid sequence similarity (Stark and Beachy, 1989; Nejidat and Beachy, 1990). However, tobacco plants expressing the AMV

coat protein were susceptible to infection from tobacco stunt virus, an *Ilarvirus*, but not to the donor strain of AMV (Bol *et al*, 1993).

The expression of viral movement proteins in transgenic plants has mostly been to investigate the role and ability to complement defective genes in the virus life cycle. Transgenic plants expressing movement protein genes have been shown to be resistant to infection (Lapidot *et al*, 1993; Cooper *et al*, 1996; Malyshenko *et al*, 1993). The movement proteins have been shown to interact with the plasmodesmata causing them to open (Waigmann *et al*, 1994; Citovsky *et al*, 1992; Angell and Baulcombe, 1995). Plants expressing transgenic movement proteins have a modified physiology such that they require constant watering and therefore are deemed unsuitable for further testing for commercial purposes.

A number of viral polymerase or replicase genes have been expressed in genetically engineered plants and in which effective resistance was obtained. These include transgenic plants containing either the entire wild-type viral replicase gene or a truncated or mutated version of; AMV (Brederode *et al*, 1995), *Tobacco mosaic virus* (Golemboski et al, 1990; Donson *et al*, 1993), *Cucumber mosaic virus* (Anderson *et al*, 1992; Zaitlin *et al*, 1994; Carr *et al*, 1994; Suzuki *et al*, 1996; Singh *et al*, 1988), *Potato virus X* (Braun and Hemenway, 1992; Longstaff *et al*, 1993), *Pea early browning virus* (Macfarlane and Davies, 1992), *Potato virus Y* (Audy *et al*, 1994), *Cymbidium ringspot virus* (Rubino *et al*, 1993; Rubino and Russo, 1995), *Pepper mild mottle virus* (Tenllado *et al*, 1995), *Tomato yellow leaf curl virus* (Brunetti *et al*, 1997), *Cowpea mosaic virus* (Sijen *et al*, 1995), *Pea seed-borne*

mosaic (Jones *et al*, 1998), *Plum pox virus* (Guo *et al*, 1998), *Rice tungro spherical virus* (Huet *et al*, 1999), *Potato leaf roll virus* (Thomas *et al,* 2000), *Wheat streak mosaic virus* (Sivamani *et al*, 2000), and *Papaya ringspot virus* (Chen *et al*, 2000).

In the cases cited above (or in subsequent studies of such), on the basis of the transgene used and if it was translatable or untranslatable, or if protein or transcript was detected, it could be suggested that the resistance observed was due to a protein mediated or (in some cases, both) RNA mediated resistance mechanism. In other studies, the expression of unmodified and/or modified replicase genes has not given rise to any resistance to viral infection. It seems that there are no clear rules as to whether or not the expression of viral replicase genes will give resistance and if they do by what means.

However, in the case of mutations to a highly conserved replicase motif, resistance was mostly via a protein mediated mechanism. The motif is GDD, which represents the amino acids, Glycine-Aspartic Acid-Aspartic Acid, which is thought to be the catalytic centre of RNA polymerase proteins (Koonin, 1991). The GDD motif is found in the 2a protein of viruses belonging to the *Bromoviridae* family (data not shown). In transgenic tobacco expressing either of the AMV 1a and 2a genes or both genes, no resistance was observed and they were shown to produce functional replicase proteins because they became infected with AMV inocula lacking the genome segments encoding the respective gene(s) in the plant (van Dun *et al*, 1988b; Taschner *et al*, 1991). In transgenic tobacco expressing the AMV 2a gene with the GDD motif changed to GGD, GVD or DDD, 20 to 30% of the transgenic lines (33

lines evaluated in total) showed a high level of resistance to infection, with the resistance being generally associated with high transcript levels (Brederode *et al*, 1995). In the same experiment when the GDD motif was changed to VDD no resistance was observed in transgenic plants even though transcript was detected.

Tobacco plants expressing the *Potato virus X* replicase protein with the GDD motif changed to ADD were resistant to viral infection (Longstaff *et al*, 1993). In the same experiment, plants expressing the gene for the replicase protein with the GDD motif changed to GAD or GED were not resistant to infection despite the same motif change being shown to render the virus non-infectious when otherwise infectious inoculum was inoculated onto plants or into protoplasts (Longstaff *et al*, 1993). In *Nicotiana benthamiana* plants expressing a gene fragment of the *Plum pox virus* containing the GDD motif that had been changed to VDD or ADD, resistance to infection by the virus was correlated with low transgene mRNA levels in these plants, suggesting that resistance was due to gene silencing (Guo *et al*, 1998).

In summary, transgenic plants expressing pathogen derived genes may or may not be resistant to infection by the source virus of the genes or to related viruses. If resistance is conferred it may be protein or RNA mediated or both. Plants expressing the CP or replicase genes typically show no adverse effects on the normal growth habit of the plant and are preferred for achieving virus resistance in commercially important crops.

3. A NEW STRATEGY TO CONFER RESISTANCE TO THE ALFALFA MOSAIC VIRUS IN PLANTS USING A MODIFICATION TO A VIRAL REPLICATION PROTEIN.

In this Section, a description is given of a the new strategy to confer resistance to AMV in plants with an explanation of the model by which it is proposed to work.

3.1. Basis for the develoment of a new genetic engineering strategy to confer resistance to plant viruses.

The new mechanism to confer resistance in plants to AMV infection developed and studied in this project involves the expression of the wildtype AMV RNA 1 gene and corresponding constructs of the same gene but which have modified ATP binding motifs so that the expressed protein can presumably no longer undertake hydrolysis of ATP. The defective AMV 1a protein is proposed to form a complex with the 2a protein which is then unable to replicate the genomic viral RNAs and thereby inhibit or slow infection by the virus.

The background and principal reasons for selecting this new strategy are discussed within this section. In brief, the reasons for selecting this strategy are:

1. Expression of pathogen derived genes involved in replication has given rise to effective virus resistance in plants, especially when a highly conserved motif in a replicase gene is mutated (Section 2.4 and 3.2);

2. The ATP binding motif has been positively identified only in the 1a protein of all tripartite viruses and is considered a highly non-random sequence, which has not been previously used. Information is available on what sequence changes to the ATP binding motif make the protein unable to undertake hydrolysis of ATP (Sections 3.3); and
3. The sequence of the 1a protein is likely to be similar between strains and so will be widely applicable to all AMV strains (Section 3.4).

3.2 Effective resistance using pathogen derived genes involved in viral replication.

The use and effectiveness of pathogen derived viral resistance genes has been previously discussed in Section 2.4. Whilst transgenic tobacco plants expressing wild type AMV 1a or 2a genes were not resistant to viral infection (Bol *et al*, 1993), tobacco plants expressing AMV 2a gene constructs that had mutations in the highly conserved GDD motif were resistant to viral infection (Brederode *et al*, 1995). This followed similar work in which the GDD motif found in the *Potato virus X* replicase protein was mutated and expressed in tobacco which gave rise to resistance to virus infection (Longstaff et al, 1993). It is proposed that the expression of the less variable (as described in Section 3.4) AMV 1a protein in the replication complex with an equally important motif (as described in Section 3.3) modified will also give rise to durable resistance.

3.3 The ATP "P-loop" binding motif - a highly conserved and mutable motif.

Essentially all the main biochemical processes including DNA replication, protein synthesis, active transport, and signal transduction are coupled to nucleoside triphosphate (NTP - usually ATP) hydrolysis (Gorbalenya and Koonin, 1989). Numerous, though not all, NTPases possess conserved amino acid sequences (Walker *et al*, 1982; Gobalenya and Koonin, 1989; Saraste *et al*, 1990). One such amino acid sequence is referred to as the Walker A motif, the NTP-binding motif but most commonly as the P-loop motif. The amino acid sequence of the motif is (A or G)XXXXGK(S or T) (where A is alanine, G is glycine, K is lysine, S is serine, T is Threonine and X can be any amino acid). The presence of this amino acid sequence suggests that the protein is involved in NTP binding as the sequence has been shown to be highly non-random and correlates well with demonstrated NTP binding or hydrolysis (Gorbalenya and Koonin, 1989; Saraste *et al*, 1990).

A second protein motif identified with association to NTP-binding is referred to as the Walker B motif or Mg^{++} binding site. The Walker B motif is hhhD(D or E) (where h is a bulky hydrophobic amino acid, D is aspartic acid and E is glutamic acid) (Gorbalenya and Koonin, 1989; Koonin, 1997). In all proteins where both the P-loop and Walker B motifs are present, the P-loop is on the amino-terminal side relative to the Walker B usually between 30 and 130 amino acids apart (Yoshida and Amano, 1995).

A motif search of all AMV protein sequences published revealed that two P-loop motifs exist in the genome. The first is in the putative helicase domain of the 1a protein and the second in the 2a protein (Figures 3 & 4). The P-loop motif was found in the 1a protein of all *Bromoviridae* viruses (Figure 3) but not in the 2a or movement proteins of the other *Bromoviridae* viruses (Figures 4 & 5). On the basis that the P-loop motif in the 1a protein is in the putative helicase domain and is located in a similar position in closely related viruses, I believe that it is highly probable to be involved in ATP binding and hydrolysis. Furthermore, for all *Bromoviridae* viruses, including AMV, a Walker B motif was identified in their 1a protein at approximately 60 amino acids from the P-loop motif on the carboxyl-terminal side (data not shown).

The P-loop motif has been mutated in the gene for the RNA-dependent RNA polymerase of *Potato virus X* where the last three amino acids of the P-loop (GKS) were changed to AKS, GNS and GES (Davenport and Baulcombe, 1997). The changes were made to infectious clones of the virus which allowed for the testing of the effect of the mutation. Clones with the AKS mutation still infected plants whilst the GNS or GES mutations did not allow virus accumulation, either in tobacco plants or protoplasts. This is consistent with previous mutational analysis of the P-loop and the idea that the lysine residue interacts with the negatively charged phosphate group of an NTP (Logan and Knight, 1993; Story *et al*, 1993; Konola *et al*, 1994).

Figure 3: ATP binding motifs identified in the 1a protein of viruses belonging to the *Bromoviridae* family

Genera	Virus	Strain	GenBank Locus or Accession #	ATP Binding motif (underlined) and location of the first amino acid of the motif in the protein
Alfamovirus	*Alfalfa mosaic virus*	425-L	MAACG1Z	838: V T I B D **G V A G C G K T** T N I K Q
	Alfalfa mosaic virus	Q	MAARNA13	Only 3' sequence
Bromovirus	*Broad bean mottle virus*		BBMIAP	690: V V M V D **G V A G C G K T** T A I K E
	Cowpea chlorotic mottle virus		MCCP1A	682: I S L C D **G V A G C G K T** T A I K S
	Brome mosaic virus		MBRCG1Z	685: I S M V D **G V A G C G K T** T A I K D
			BRBMV1	685: I S M V D **G V A G C G K T** T A I K D
			BMV1APROT	685: I S M V D **G V A G C G K T** T A I K D
Cucumovirus	*Cucumber mosaic virus*	Y	D12537	715: I S Q V D **G V A G C G K T** M P I K S
		Q	CURNA1Q	713: I S Q V D **G V A G C G K T** T A I K S
		Fny	MCVFRNA1	714: I S Q V D **G V A G C G K T** T A I K S
			CMU20220	714: I S Q V D **G V A G C G K T** T A I K S
			MCVR1PB*	99: I S Q V D **G V A G C G K T** T A I K S
		lizuka	MCVL1	714: I S Q V D **G V A G C G K T** T A I K S
	Tomato aspermy virus	V	TOAVRNA1	714: I S L V D **G V A G C G K T** T A I K K
	Peanut stunt virus	J	PSVJ1A	722: I S L V D **G V A G C G K T** T A I K K
Ilarvirus	*Tobacco streak virus*		AAB48983	806: I T I V D **G V A G C G K T** T H L K K
	Citrus leaf rugose virus		CLU23715	765: V I I E D **G V A G C G K T** T S L L K
	Elm mottle virus		SLU57047	774: V V I E D **G V A G C G K T** T S L L K
	Spinach latent virus		PMOVRNA1	775: I V I E D **G V A G C G K T** T S L L K
	Prune dwarf virus		PDU57648	770: I T I M D **G V A G C G K T** T K I K S
Oleavirus	*Olive latent virus 2*		OLV21APRT	631: K T W I D **G V A G C G K T** Y E I V H

*NOTE: The sequence of the *Cucumber mosaic virus* entry MCVR1PB was only the 3' end of the 1a gene.

Figure 4: ATP binding motifs identified in the 2a protein of viruses belonging to the *Bromoviridae* family

Genera	Virus	Strain	GenBank Locus or Accession #	ATP Binding motif (underlined) and location of the first amino acid of the motif in the protein
Alfamovirus	*Alfalfa mosaic virus*	425-L	MAACG2Z	747: A L E S L **G K I F A G K T** L C K E C
			A1MVRNA2	747: A L E S L **G K I F A G K T** L C K E C
Bromovirus	*Broad bean mottle virus*		BBMRNA2Q	no ATP binding motif
			BBU24495	no ATP binding motif
		Mo	BBU24496	no ATP binding motif
	Cowpea chlorotic mottle virus		MCCRNAA2	no ATP binding motif
	Brome mosaic virus		MBRCG2Z	no ATP binding motif
			BRBMV2	no ATP binding motif
			BMV2APROT	no ATP binding motif
Cucumovirus	*Cucumber mosaic virus*	Fny	MCVRN2	no ATP binding motif
		Y	D12538	no ATP binding motif
		Q-CMV	CVRNA02	no ATP binding motif
		NT9	MCV2A2	no coding region defined
			MCVORNA2	no ATP binding motif
			MCVL2	no ATP binding motif
	Tomato aspermy virus	V	TOAVRNA2	no ATP binding motif
	Peanut stunt virus	J	PSVJ2A	no ATP binding motif
Ilarvirus	*Tobacco streak virus*		TSU75538	no ATP binding
	Citrus leaf rugose virus		CLU17726	no ATP binding
	Elm mottle virus		SOU34050	no ATP binding
	Spinach latent virus		PMOVRNA2	no ATP binding
	Prune dwarf virus		AF277662	no ATP binding
Oleavirus	*Olive latent virus 2*		OLV22APRT	no ATP binding

Figure 5: ATP binding motifs identified in the 3a protein of viruses belonging to the *Bromoviridae* family

Genera	Virus	Strain	GenBank Locus or Accession #	ATP Binding motif (underlined) and location of the first amino acid of the motif in the protein
Alfamovirus	*Alfalfa mosaic virus*	425-S	ALAM19	no ATP binding motif
		YSMV	MAA32KDMP	no ATP binding motif
		425-M	MAACG3Z	no ATP binding motif
		3-L	MAARNA3L	no ATP binding motif
Bromovirus	*Broad bean mottle virus*		BBM3ACT	no ATP binding motif
	Cowpea chlorotic mottle virus		MCCRNA3	no ATP binding motif
			MCCRNAA3	no ATP binding motif
	Brome mosaic virus	Russian	MBRCG3Z	no ATP binding motif
			BRBMV3	no ATP binding motif
			BMV3APROT	no ATP binding motif
Cucumovirus	*Cucumber mosaic virus*	Q	MCVRNA3A	no ATP binding motif
		trk 7	MCV3APCOAT	no ATP binding motif
		O	MCVO3	no ATP binding motif
		Kor	MCVRNA3KOR	no ATP binding motif
		Y	MCVRNA3	no ATP binding motif
			CMV3ACP	no ATP binding motif
		WL	MCVRNA3WL	no ATP binding motif
		C	MCVRNA3C	no ATP binding motif
			CMU37227	no ATP binding motif
			MCV3APA	no ATP binding motif
			CMU20219	no ATP binding motif
		E5	MCVR3MPCP2	no ATP binding motif
			CMU20668	no ATP binding motif
		C7-2	MCVST3ACP	no ATP binding motif
	Tomato aspermy virus	C	TOARNA3	no ATP binding motif
		P	TOA3APCOAT	no ATP binding motif
	Peanut stunt virus	J	PSVRNA3	no ATP binding motif

continued next page

Figure 5 continued: ATP binding motifs identified in the 3a protein of viruses belonging to the *Bromoviridae* family

Genera	Virus	Strain	GenBank Locus or Accession #	ATP Binding motif (underlined) and location of the first amino acid of the motif in the protein
Ilarvirus	*Tobacco streak virus*		TOTSV3	no ATP binding
	Citrus leaf rugose virus		CLU17390	no ATP binding
	Elm mottle virus		SLU57048	no ATP binding
			EMU85399	no ATP binding
	Spinach latent virus		PMOVRNA3	no ATP binding
	Prune dwarf virus		PDVMOVCAP	no ATP binding
		ch 137	PDVMOVCAP	no ATP binding
	Apple Mosaic Virus		AMU15608	no ATP binding
	Hydrangea mosaic virus		HMU35145	no ATP binding
Oleavirus	*Olive latent virus 2*		OLV212	no ATP binding

3.4 Sequence similarity in the 1a protein between strains of the alfalfa mosaic virus.

Two lines of evidence suggest that the sequence of RNA 1 does not vary significantly between strains of AMV.

The first is by the evaluation of pseudorecombinant strains – such strains are formed by mixing the genomic RNAs from different strains of the virus, for example RNA 1 from strain A with RNAs 2-4 from strain B. By purifying the genomic RNAs from two strains of AMV with distinctly different hosts and recombining them to form pseudorecombinant viruses, RNA 1 was shown to not confer differences of symptoms on tobacco or french bean (*Phaseolus vulgaris*) (Dingjan-Versteegh *et al*, 1972). In the same research RNA 2 was shown to confer symptom characteristics in french bean which are used for subgroup classification of AMV strains (Dingjan-Versteegh *et al*, 1972).

The second line of evidence is based on restriction fragment length polymorphism analysis. Research in New Zealand has shown that cDNA clones of RNA 1 from different AMV strains have the same restriction enzyme recognition sites, whilst the same analysis of clones from RNA 2 suggests that it is highly variable between strains (Richard Forster, pers. comm.).

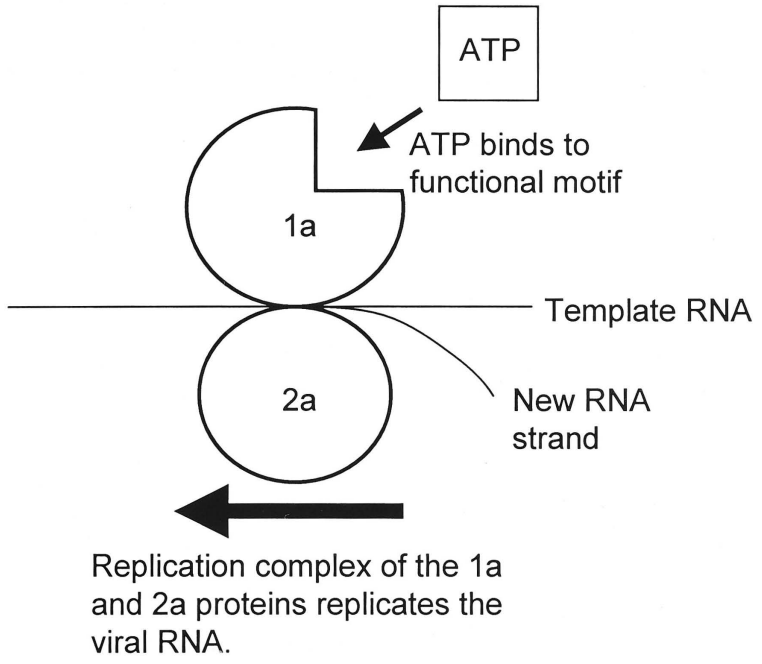
3.5 Model for the mechanism of viral resistance using the new strategy.

Two models are proposed for the mechanism by which the genes with mutated P-loops could confer resistance to viral infection.

The first model is a protein mediated mechanism by which the 1a protein molecules defective in ATP binding bind with 2a protein molecules to form dysfunctional viral replication complexes (Figure 6). With a large number of defective 1a molecules present in the cytoplasm of cells, 2a protein molecules synthesized by infecting virus should not be able to form functional replication complexes and thereby stop further viral replication and infection.

The second model is an RNA mediated mechanism as described in Section 2.3. Whilst the mechanisms of RNA mediated resistance are not fully understood, when expressing the full length of an otherwise unmodified gene a high level of transcript message is required for resistance to be achieved (Flavell, 1994; Goodwin *et al*, 1996). The best way to achieve durable RNA mediated resistance would be through high levels of transgene expression, such as having several copies of the transgene (Goodwin *et al*, 1996). The RNA mediated mechanism is not a focus of this research project as such resistance is often limited to viral strains with close sequence similarity (Mueller *et al*, 1995; van den Boogaart *et al*, 1998).

A) Virus replication by wild type AMV 1a protein.



B) Virus replication inhibited by mutant AMV 1a protein.

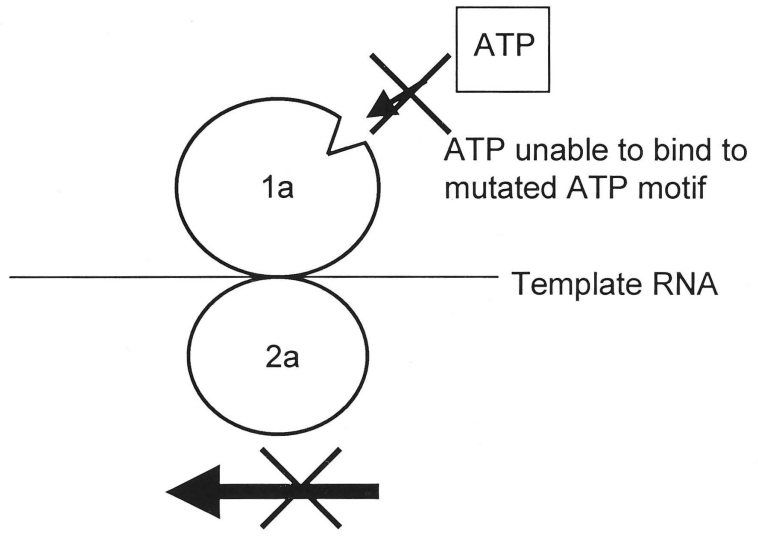


Figure 6: Model for protein mediated viral resistance by the expression of AMV 1a protein with a mutated and defective ATP binding motif in transgenic plants. In wild type plants (as in A), virus replication is carried out by the replication complex formed by AMV 1a and 2a proteins hydrolyzing ATP. In transgenic plants (as in B), mutant AMV 1a disrupts the replication of the viral RNA by not being able to hydrolyze ATP.

CHAPTER 2 DEVELOPMENT AND ANALYSIS OF ALFALFA MOSAIC VIRUS RNA 1 ATP BINDING MOTIF MUTANTS

1. INTRODUCTION.

The first step in generating transgenic plants expressing AMV 1a proteins with dysfunctional ATP binding motifs is to be certain that the putative motif is involved in ATP hydrolysis and that the proposed mutations make it dysfunctional. The strategy pursued to demonstrate that the putative ATP binding site in the AMV 1a protein is presumably involved in ATP hydrolysis and that the mutations proposed to the motif make it dysfunctional was to make AMV RNA 1 infectious clones with the respective mutations and then to test their infectivity.

All four genomic AMV RNAs (1-4) from strain 425 have been cloned into pUC 9 based vectors with a 35S promoter and a *nos* terminator (pCa17T, pCa27T, pCa32T and pCa42T, respectively for RNA 1, 2, 3 and 4), and have been shown to be infectious when they are all co-inoculated onto *Nicotian tabacum cv* Samsun NN (Neeleman *et al*, 1993). The infectious clones can be thought of as cDNA clones which have the 35S promoter and a *nos* terminator. In this chapter, the process by which the mutant AMV RNA 1 infectious clones were made and evaluated *in vivo* is described. The evaluation of the model for conferring viral resistance in plants by the expression of defective interfering AMV 1a proteins is also described.

2. MATERIALS AND METHODS.

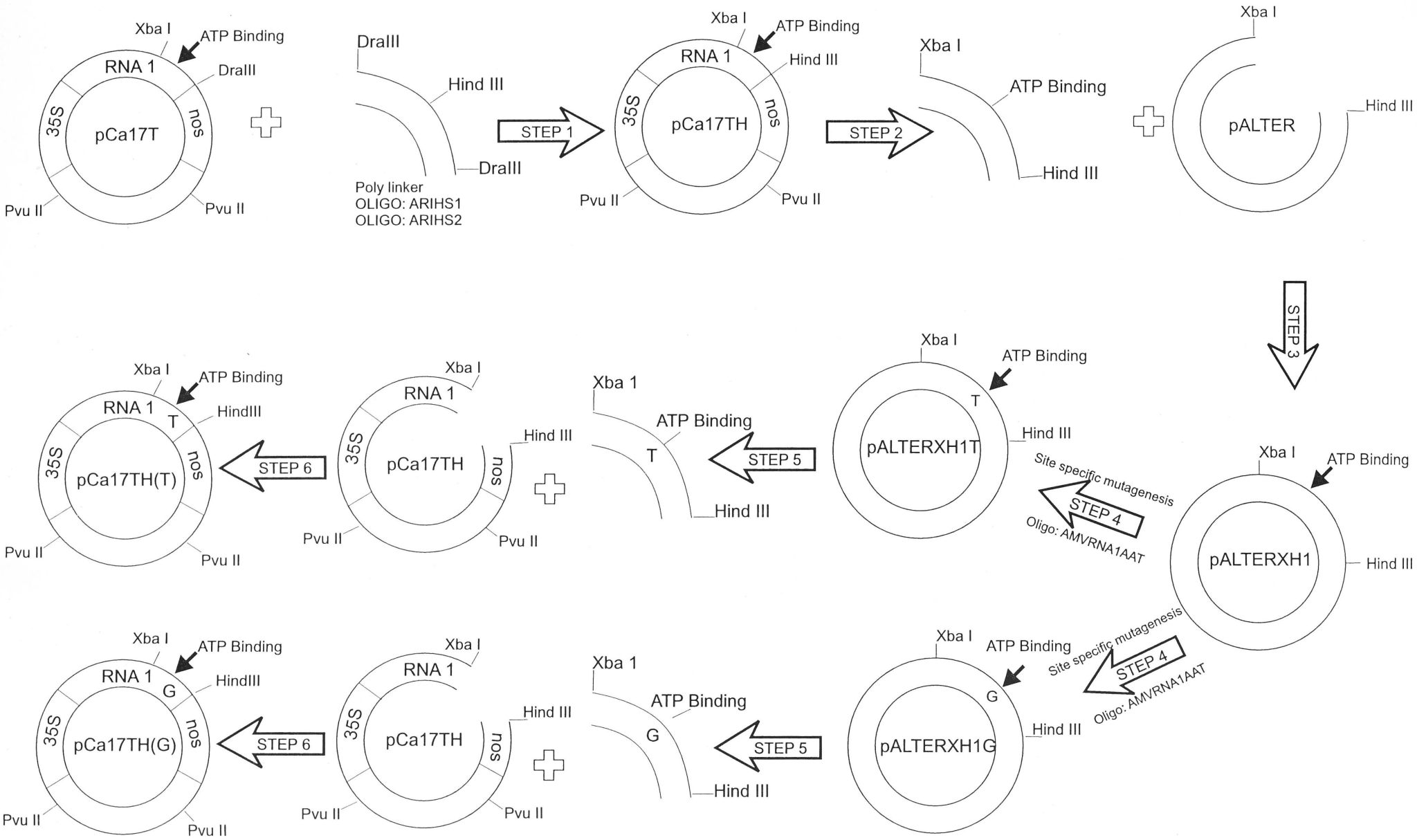
2.1 General Materials and Methods.

All nucleic acid preparation, cloning and transformation procedures were carried out according to standard laboratory practice essentially as described by Sambrook *et al*, 1989. Only analytical or molecular biology grade reagents, enzymes and biochemicals were used. All plasmid transformations unless otherwise indicated were carried out using *E.coli* strain DH5α. All plants were propagated in pots containing a recycled steam sterilised compost mix originally prepared from a loamy soil (33%), peat moss (33%) and vermiculite (33%) mix. The plants were grown in a sealed glasshouse without the aid of additional lighting but with the temperature maintained at approximately 16°C during the night and 23°C during the day.

2.2 Cloning strategy for the development of AMV RNA 1 infectious clone mutant deriviatives with defective ATP binding.

The cloning strategy for the development of the AMV RNA 1 infectious clones with defective ATP binding will now be described, it is also summarized in Figure 7. The AMV RNA 1 infectious clone pCa17T was digested with the *DraIII* restriction enzyme and a polylinker with oligonucleotides (AR1HS1 and AR1HS2) were ligated with T4 DNA ligase (Promega) at a 500:1 molar ratio of oligonucleotide to plasmid DNA (30ng). The sequence of the AR1HS1 and AR1HS2 oligonucleotides are 5' GTGAAGCTTCCCGGGCACTGG 3' and

Figure 7: Diagram of the cloning strategy to produce the infectious and mutant derived plasmid clones pCa17TH, pCa17TH(G) and pCa17TH(T).


Xba I
ATP Binding
RNA 1
DraIII
35S
pCa17T
nos
Pvu II
Pvu II
DraIII
Hind III
DraIII
Poly linker
OLIGO: ARIHS1
OLIGO: ARIHS2
STEP 1
Xba I
ATP Binding
RNA 1
Hind III
35S
pCa17TH
nos
Pvu II
Pvu II
STEP 2
Xba I
ATP Binding
Hind III
Xba I
pALTER
Hind III
STEP 3
Xba I
ATP Binding
T
Hind III
pALTERXH1T
Site specific mutagenesis
STEP 4
Oligo: AMVRNA1AAT
Xba I
ATP Binding
pALTERXH1
Hind III
Xba 1
ATP Binding
T
STEP 5
Hind III
Xba I
RNA 1
35S
pCa17TH
nos
Hind III
Pvu II
Pvu II
STEP 6
Xba I
ATP Binding
RNA 1
T
HindIII
35S
pCa17TH(T)
nos
Pvu II
Pvu II
Xba I
ATP Binding
G
Hind III
pALTERXH1G
Site specific mutagenesis
STEP 4
Oligo: AMVRNA1AAT
Xba 1
ATP Binding
G
STEP 5
Hind III
Xba I
RNA 1
35S
pCa17TH
nos
Hind III
Pvu II
Pvu II
STEP 6
Xba I
ATP Binding
RNA 1
G
HindIII
35S
pCa17TH(G)
nos
Pvu II
Pvu II

5' GTGCCCGGGAAGCTTCACCCA 3' respectively. The polylinker introduced one *HindIII* and one *SmaI* restriction enzyme site so as to allow the DNA coding sequence for the ATP binding motif could be cloned into the site specific mutagenesis vector p-ALTER-1 (supplied by Promega). The plasmid formed is called pCa17TH.

pCa17TH plasmid was then digested with *XbaI* and *HindIII* restriction enzymes with the resultant fragment containing the DNA sequence coding for the ATP binding motif cloned into *XbaI* and *HindIII* digested pALTER-1 to produce the plasmid pALTERXH1. Site specific mutagenesis was undertaken using Promega Altered Sites®II *in vitro* Mutagenesis System (as described in Section 2.3). Two plasmids containing the required changes to the DNA sequence coding for the ATP binding motif were produced, called pALTERXH1G and pALTERXH1T – the last letter in the name of these plasmids refers to the DNA base changed. The mutagenesis was confirmed by sequencing (as described in Section 2.4). The pALTERXH1G and pALTERXH1T plasmids were digested with *XbaI* and *HindIII* and the respective DNA fragment containing the sequence for the now mutated ATP binding motif were re-cloned back into pCa17TH to produce the plasmids pCa17TH(G) and pCa17TH(T). Sequence analysis confirmed the mutant forms.

2.3 Site specific mutagenesis.

Site specific mutagenesis was undertaken according, but with some modifications, to the instructions of the Promega Altered Sites®II *in vitro* Mutagenesis System

protocol. The system involves denaturing the pALTERXH1 plasmid into single strands and then two oligonucleotides, one to repair the ampicillin resistance gene and one to confer the desired change in the DNA sequence, were used to make a copy containing the desired sequence change which can be more probably identified by positive selection for ampicillin resistance.

Alkaline denaturation of the plasmid pALTERXH1 was achieved by adding 0.2µg of the plasmid DNA to a 2µl solution of 2M NaOH and 2M EDTA and then making this mixture up to 20ul with dH_2O. The solution was then incubated at room temperature for 5 minutes after which 2µ of 2M ammonium acetate (pH 4.6) and 75µl of ethanol (100%) was added and the DNA precipitated at -70°C for 30 minutes. Precipitated DNA was recovered by centrifuged (10,000 g) for 15 minutes and washed with 200µl of 70% ethanol. The pellet was resuspended in 10µl of TE buffer (pH 8.0) in preparation for the annealing reaction.

Oligonucleotides (21 nucleotides) were designed for the site specific mutagenesis of the ATP binding site. The sequences designated AMVRNA1GAA (for changing a codon from AAA to GAA), and AMVRNA1AAT (for changing a codon from AAA to AAT) are given in Figure 8. The mutagenesis mixture consisted of 0.05pmol of the ss template, 0.25pmol of the ampicillin repair oligonucleotide, 2.5pmol of the mutagenic oligonucleotide and 2µl of the 10X annealing buffer supplied were made up to a total volume of 20µl with dH_2O. Annealing was achieved by heating the mixture to 70° for 5 minutes and then allowed to cool to room temperature before placing on ice. To this mixture 3µl of the 10X synthesis buffer, 10U of T4 DNA

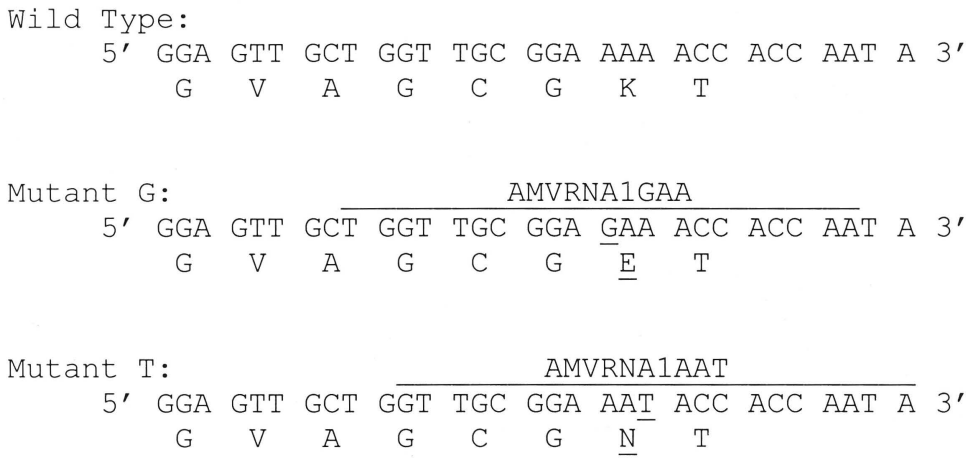


Figure 8: DNA and protein sequence of the putative ATP binding motif in wild type and mutant AMV RNA 1 and the oligonucleotides used for the site specific mutagenesis. The sequence of the oligonucleotides (AMVRNA1GAA and AMVRNA1AAT) used for the site specific mutagenesis is indicated by the line above the DNA sequence. The T and G DNA base change is indicated by an underline as is the resultant amino acid change. The change in Mutant G is referred in the thesis as being the 'G' series from the base changed, similarly Mutant T is referred to as the 'T' series.

polymerase, 2U of T4 DNA ligase were added and then made up to a volume of 30µl with dH_2O and then mixed before incubation at 37°C for 90 minutes to complete the synthesis of dsDNA containing the required base changes.

E.coli strain ES1301 *mutS* was transformed by taking 100µl of competent cells and adding 3µl of DMSO, 100ng of R408 helper phage DNA and the reaction mixture (30µl) and then incubating on ice for 30 minutes before heat shocking the cells at 42°C for 2 minutes. To the cells, 4ml of LB medium without antibiotic was added and then incubated at 37°C for 5 hours. The cells were then centrifuged (10,000 g) and resuspended in 100µl of an overnight culture (without antibiotic) of *E.coli* strain JM109 which were incubated for 3 hours at 37°C before plating onto LB media containing 100µg/ml of ampicillin. Single colonies containing the mutant plasmids were then selected for plasmid purification (miniprep) and sequence analysis (as described in Section 2.4) to ensure the desired mutation had been incorporated.

2.4 DNA sequencing.

DNA sequencing was carried out in plasmid DNA using the ABI Prism Dye Terminator Cycle Sequencing System (part# 402078) manufactured and supplied by Perkin Elmer with substantial modification to the suggested protocol. The key elements to the protocol are the preparation of template DNA by precipitation with PEG 8000 and the use of 5pmol of primer per reaction.

Cells containing the plasmid of interest for sequencing were grown overnight at 37°C in 3ml of LB media with an appropriate antibiotic and then centrifuged. The pellet was resuspended in 100µl of GTE solution and 200µl of freshly prepared lysis solution (0.2M NaOH and 1% SDS) was added and mixed by gentle inversion. Following incubation on ice for 5 minutes, 150µl of 3M KAc was added and mixed by gentle inversion before incubation on ice for 10 minutes followed by centrifugation. The supernatant was collected and two volumes of absolute ethanol was added and then mixed. After centrifugation at room temperature for 5 minutes the supernatant was removed and the pellet washed with cold 70% ethanol followed by centrifugation for 2 minutes at room temperature. The pellet was air dried and then resuspended in 70.2µl of TE with 50µg/ml of RNase A and incubated at room temperature for 30 minutes. To the mixture 12.8µl of 5M NaCl and 80µl of 13% w/v PEG 8000 was added and mixed by gentle inversion and incubated on ice for 20 minutes before centrifugation for 15 minutes at 4°C. The pellet was resuspended in 100µl of TE to which 100µl of phenol and 100µl of chloroform was added, then vortexed and then centrifuged (10,000 g) for 5 minutes. The aqueous (upper) phase was collected and $1/10^{th}$ volume of 3M NaOAc (pH 5.2) and two volumes of ethanol (100%) added before incubation at -20°C for at least 60 minutes. The sample was then centrifuged (10,000 g) for 30 minutes at 4°C with the pellet air dried and resuspended in 20µl of TE.

The sequencing reaction utilised 8.0µl of the 'Terminator ready reaction mixture' to which 500ng of template DNA and 5.0pmol of primer were mixed and made up to a volume of 20µl with dH_2O. Each reaction was loaded into a PCR capillary tube and

the following temperature sequence was used; 96°C for 30 seconds, 50°C for 15 seconds, 60°C for 4 minutes, with the sequence repeated for 25 cycles. After PCR, to each reaction 2.0µl of 3M NaOAc (pH 5.2), 47.5µl of ethanol (100%) and 2.5µl of dH_2O was added, vortexed and placed on ice for 10 minutes. The mixture was centrifuged at 4°C for 10 minutes with the pellet washed with 500µl of cold 70% ethanol before centrifugation at 4°C for 10 minutes. The pellet was vacuum dried in preparation for loading into the sequencer.

The DNA sequence coding for the ATP binding motif was always confirmed in putative clones in both the forward and reverse directions using the primer AMV1ATPFP (5' GTCTTTGTTGACCAATCTTGCGTC 3' and the primer AMV1ATPRP (5' AACTTTGTCAACGGTGAACAATCG 3') respectively. The AMV1ATPFP primer binds at a position 80 nucleotides to the 5' side of the sequence coding for the ATP binding motif and the AMV1ATPRP binds at a position 95 nucleotides to the 3' side.

2.5 Large scale plasmid preparation.

Large quantities of the plasmid DNA of the infectious clones and derivatives of such for the purpose of inoculation onto plants were prepared using a Maxi Plasmid Preparation Kit (catalogue # 12163) manufactured by Qiagen. The only variation to the standard procedure was that 2L of culture was harvested rather than the recommended 500ml due to the slow growth rate of the bacteria and low yield of plasmid DNA (typically 20µg per L of culture). All of the plasmids were grown in

E.coli strain DH5α. The quantity and quality of DNA was determined by spectrophotometer and agarose gel electrophoresis.

2.6 Inoculation and infectivity evaluation of the infectious and mutant derivative clones

The plasmid DNA of each infectious and derivative clone was digested separately with *PvuII* which cleaves at positions 200bp upstream of the 35S promoter and 90bp downstream of the *nos* terminator (Neeleman *et al*, 1993; see also Figure 7). The restriction enzyme was heat inactivated at 65°C for 30 minutes. Complete digestion was confirmed by agarose gel electrophoresis and the quantity of DNA was estimated by measuring the absorbance at 260nm. Mixtures of the infectious clones to give the appropriate amount of each digested plasmid were made prior to inoculation and were verified by gel electrophoresis.

Cowpeas (*Vigna unguiculata,* cultivar Blackeye) were inoculated when the first leaves reached full expansion which ranged from 4 to 6 six days after germination in the glasshouse. Only plants with uniform growth and no emerging shoot tips were used. The selected seedlings were sensitised to virus infection by being placed in the dark for about 16 hours before inoculation. A light sprinkling of 37μm carborundum was placed onto each half leaf immediately before a water mixture (20μl) of the plasmids of the infectious clones and derivatives was applied. The leaves were gently rubbed five times. After a period of 5 to 15 minutes, the inoculated leaves

were washed with water. Local lesions were assessed and counted between 4 and 7 days after inoculation.

2.7 Preparation of AMV strain WC28 viral inoculum.

A white clover plant infected with AMV isolate WC28 (a single lesion isolate from white clover grown at Glen Innes, New South Wales, Australia, which gives rise to severe symptoms on cowpeas and white clover, Paul Chu pers. comm.) was maintained in the glasshouse. Viral inoculum was obtained by harvesting leaves showing strong symptoms of infection and with the sap extracted in 5 or 10 volumes (as required) (w/v) 0.1M phosphate buffer (pH 7.4) using a sap extractor. At least 1g of tissue was used for each extraction.

3. RESULTS.

3.1 Comparison of local lesions on cowpeas inoculated with AMV isolate WC28 and different levels of the AMV infectious clones.

Since inoculation of AMV infectious clones had not been reported on cowpeas, I determined the optimum amount of each clone DNA for inoculation so that infectivity could be quantitatively assessed by the number of local lesions. The infectious clones (pCa17T, pCa27T, pCa32T and pCa42T which code for the AMV genomic RNAs 1, 2, 3 and 4 respectively) were inoculated onto the half leaves of

cowpeas at three different levels (0.5μg, 2.0μg and 10μg of each construct), with four replicates, and using AMV isolate WC28 viral inocula as a positive control.

The results are given in Figure 9. Inoculations using 2.0μg of each infectious clone gave around five times the number of local lesions as the 0.5μg but a similar number to that where 10μg of each infectious clone was inoculated. Cowpeas inoculated with 50μl of 1:10 w/v dilution of AMV isolate WC28 inoculum gave around 100 lesions per half leaf.

The infectious clones gave rise to local lesions typical of that caused by AMV although they were in general smaller in size than those caused by AMV isolate WC28 (Figure 10). Single lesions from both the infectious clones and AMV isolate WC28 were re-inoculated onto cowpeas to confirm that they were indeed caused by virus infection. All but the very smallest of lesions (less than 1mm in diameter) gave rise to lesions for both the infectious clones and AMV isolate WC28. This was presumably due to a deficiency in the amount of virus inocula that could be extracted from the very small lesions. The appearance of the lesions in the re-inoculation were the same as for those on the initial inoculated leaves. In the negative control, no lesions were observed where water or 0.1M phosphate buffer (pH 7.4) was inoculated. I speculate that the differences in the size of the local lesions resulting from AMV strain WC28 compared to the infectious clones may be a result of differences in the strains rather than the nature of the 'inocula'.

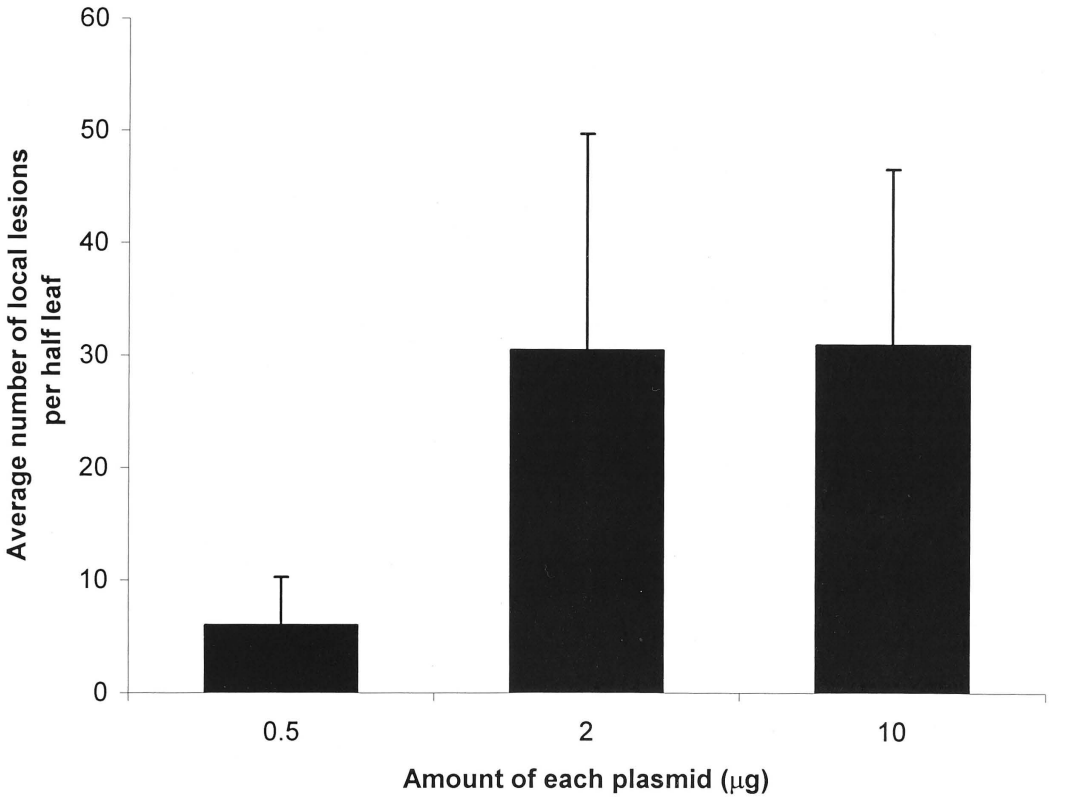
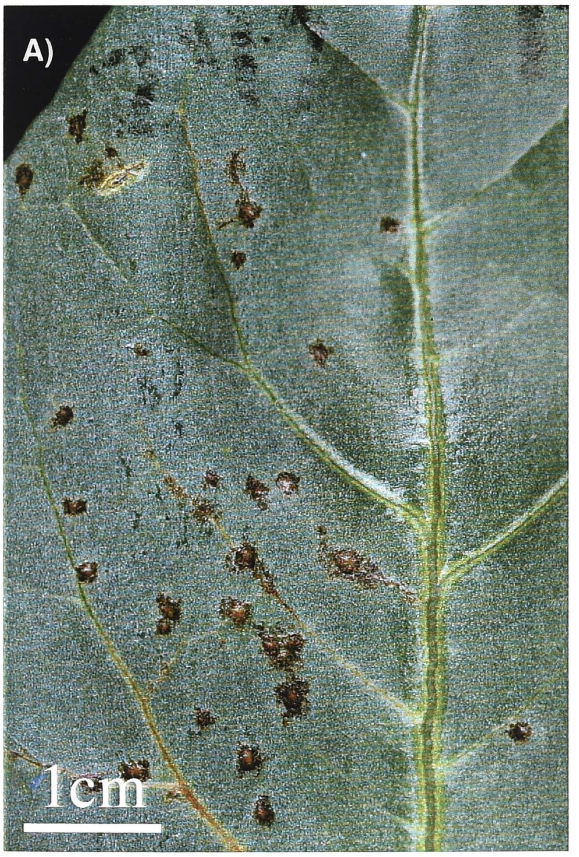


Figure 9: Mean number of local lesions per half leaf (+ s.d.) on cowpeas following inoculation with different amounts (0.5μg, 2.0μg and 10μg) of each of the four AMV infectious clones (RNAs 1-4). Four replicates were inoculated for each treatment.

Figure 10: (A) Cowpea half leaves inoculated with 50µl of 1:10 w/v dilution of AMV strain WC28 viral inoculum and (B) 2.0µg of each of the AMV infectious clones, pCa17T, pCa27T, pCa32T and pCa42T. The average lesion size resulting from inoculation with viral inoculum was in general greater than that of the AMV infectious clones. In all other respects, the lesions were typical of that caused by AMV infection.


A)
1cm


B)
1cm

3.2 Comparison of the infectivity of unmodified to modified AMV RNA 1 infectious clones.

The infectivity of the wild type AMV RNA 1 infectious clone pCa17T was compared with the three made as described above, pCa17TH, pCa17TH(G) and pCa17TH(T), by inoculating separately 2μg each of the plasmids with 2μg of each of the other infectious clones required for viral infection (pCa27T, pCa32T and pCa42T) representing AMV RNAs 2-4. Four replicate cowpea half leaves were inoculated in two separate experiments. The results of each experiment were comparable and the results pooled, and are shown in Figure 11.

The infectious clone with the insertion of the polylinker sequence in the 3' untranslated region (pCa17TH) had a 50% reduction in infectivity compared to the 'wild type' (pCa17T). Both plasmids which contained the modified ATP binding motif (pCa17TH(G) and pCa17TH(T)) did not give rise to any local lesions and therefore were deemed to be non-infectious.

3.3 Comparison of the infectivity of unmodified to modified AMV RNA 1 infectious clones at different concentrations with different levels of AMV RNAs 2-4 infectious clones.

To investigate if the lower infectivity of the altered forms of the AMV RNA 1 infectious clones, as observed in the results of Section 3.2, could be compensated by inoculating with increasing amounts of the clones with the same or higher levels of

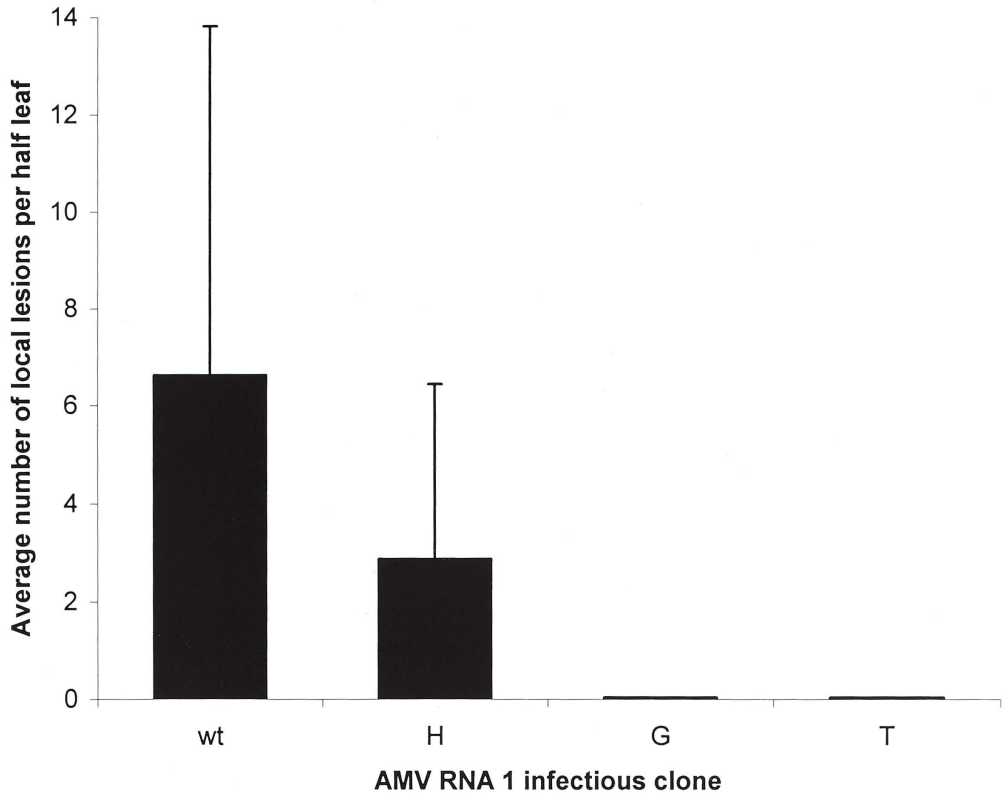


Figure 11: Mean number of local lesions per half leaf (+s.d.) on cowpeas following inoculation with 2.0μg of either pCa17T {wt}, pCa17TH {H}, pCa17TH(G) {G} or pCa17TH(T) {T} along with 2.0μg of each of the AMV RNA 2-4 infectious clones. Eight replicates were inoculated for each AMV RNA 1 infectious clone combination. All plasmids were digested with the restriction enzyme *PvuII* prior to inoculation.

the DNA representing RNAs 2-4, a number of inoculations were undertaken with different combinations of the infectious clones. Four inoculations with four combinations for each of the AMV RNA 1 infectious or mutant derived clones were undertaken, at three levels (2μg, 5μg and 10μg) with either of 2μg or 5μg of each of the AMV RNA 2-4 clones. For each combination, four replicate cowpea half leaves were inoculated. The results are given Table 1 and summarized in Figure 12.

With regard to the unmodified AMV RNA 1 infectious clone (pCa17T), the number of lesions increased with the amount of DNA inoculated and with the amount of the RNA 2-4 infectious clones. With regard to the pCa17TH infectious clone, the level of infectivity was reduced with increasing amounts of the plasmid DNA inoculated. This was exacerbated with increasing amounts of the RNAs2-4 infectious clones. In contrast, the RNA 1 infectious clones with the modified ATP binding motif (pCa17TH(G) and pCa17TH(T)) gave no local lesions regardless of the amount of DNA inoculated at either of two levels of DNA for the RNA 2-4 infectious clones.

Given the small data set, two-way analysis of variance statistical tests were carried out in a way described by Siegel, 1956. There was a significant difference at the 5% level ($p<.001$) between the four treatments using the pCa17T (wt) plasmid and the treatments using the pCa17TH (H) plasmid. Similarly, there was a significant difference at the 5% level ($p<0.001$) between the inoculations using the pCa17TH (H) and each inoculation using the mutant derivative clones pCa17TH(G) (G) and pCa17TH(T) (T). There was no difference at the 5% level between different inoculations using the same AMV RNA 1 derived plasmid ($p=0.811$). The same

TABLE 1: The number of local lesions on cowpea half leaves inoculated with various combinations in the amount of the AMV infectious and mutant derived clones.

Mixture name	Amount of pCa17T per half leaf (μg)	Amount of pCa17TH per half leaf (μg)	Amount of pCa17TH(G) per half leaf (μg)	Amount of pCa17TH(T) per half leaf (μg)	Amount of each RNA 2-4 infectious clone per half leaf. (μg)	Number of local lesions. Mean ±s.d.
A(wt)	2				2	4.0 ± 4.1
B(wt)	5				5	7.8 ± 3.5
C(wt)	10				2	5.5 ± 3.3
D(wt)	10				5	9.0 ± 5.6
A(H)		2			2	3.5 ± 5.2
B(H)		5			5	1.5 ± 1.0
C(H)		10			2	1.0 ± 0.8
D(H)		10			5	0.8 ± 1.0
A(G)			2		2	0
B(G)			5		5	0
C(G)			10		2	0
D(G)			10		5	0
A(T)				2	2	0
B(T)				5	5	0
C(T)				10	2	0
D(T)				10	5	0

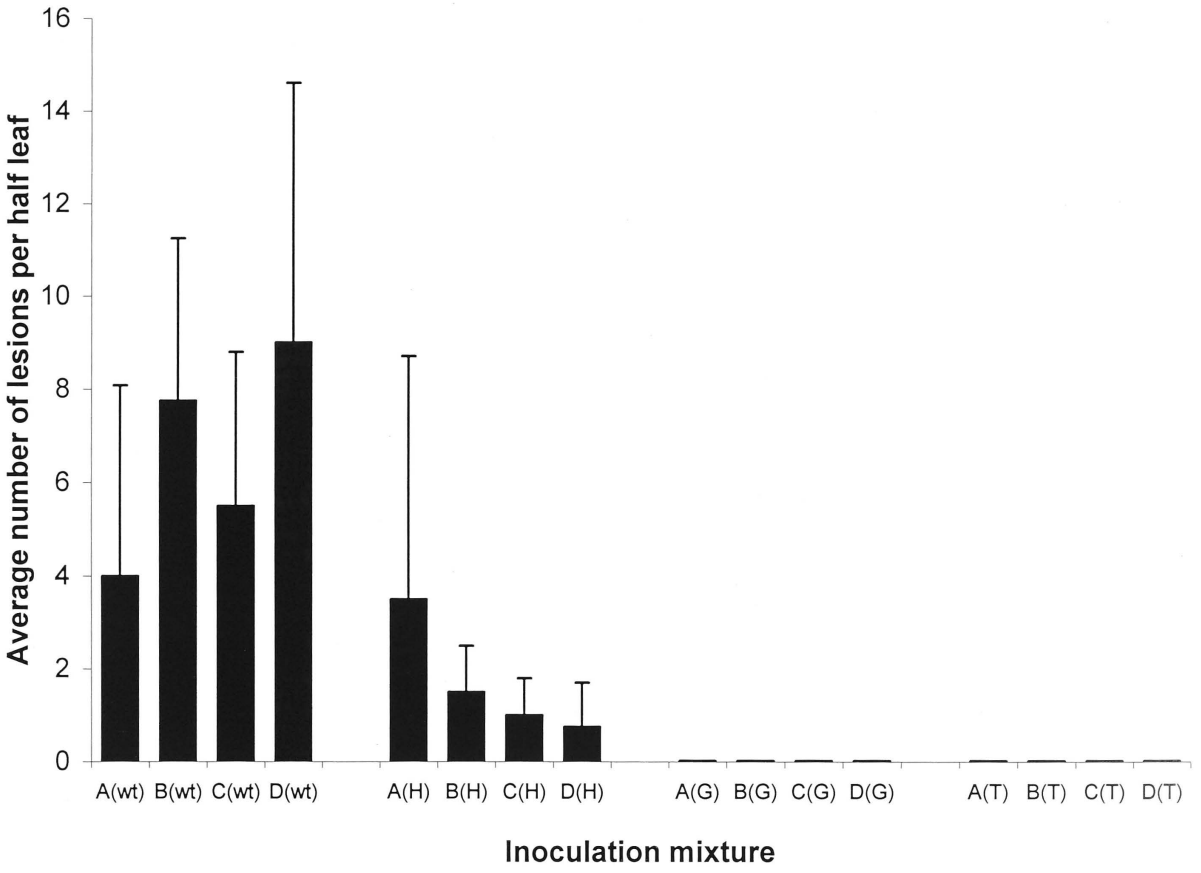


Figure 12: Mean number of lesions per half leaf (+s.d.) on cowpeas following inoculation with various combinations in the amount of the AMV infectious clones. Letters A to D each represent a different combination of the infectious clones. Letter A represents inoculation with 2μg of the RNA 1 construct with 2μg of each of the RNA 2-4 constructs; B represents 5μg of the RNA 1 construct with 5μg of each of the RNA 2-4 constructs; C represents 10μg of the RNA 1 construct with 2μg of each of the RNA 2-4 constructs; and D represents 10μg of the RNA 1 construct with 5μg of each of the RNA 2-4 constructs. The letters in the brackets represent the RNA 1 construct used: pCa17T (wt); pCa17TH (H); pCa17TH(G) (G); and pCa17TH(T) (T).

statistical significance outcomes were also achieved using the non-parametric Mann-Whitney U test.

It appears that the insertion of the polylinker in the 3' untranslated region of AMV RNA 1 reduces infectivity which is further reduced with increasing amounts of the construct or the amount of RNAs 2-4. The constructs with the modified ATP binding sites were not infectious regardless of the amount of DNA inoculated or the amount of the RNA 2-4 plasmid DNA. This confirms the results of the previous experiment that the constructs pCa17TH(G) and pCa17TH(T) were not infectious. It can be concluded that the putative ATP binding motif in the AMV RNA 1 gene is essential for virus infection and that the mutations made to it have completely disrupted its activity.

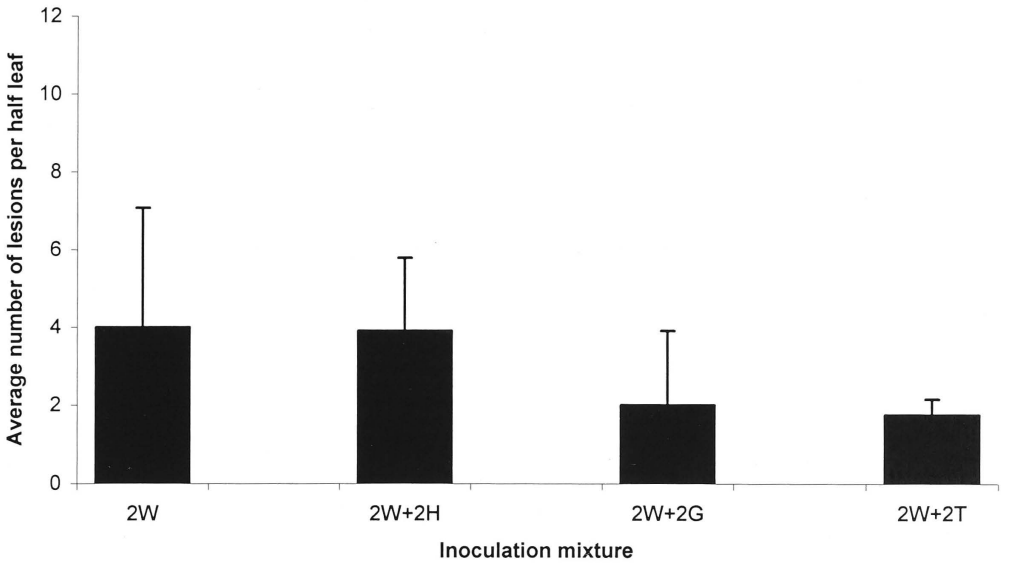
3.4 Competitive inhibition of virus infection by mutant forms of the AMV RNA 1 infectious clone.

To test if AMV 1a protein derived from the mutant forms of the AMV RNA 1 infectious clones that are presumably defective for ATP binding could inhibit *in vivo* the infection of AMV, mixtures of the unmodified (2μg) and modified (2μg or 10μg) AMV RNA 1 infectious clones were co-inoculated onto cowpea half leaves with 2μg of each of the AMV RNAs 2-4 infectious clones. The results are given in Table 2 and are summarized in Figure 13.

TABLE 2: The number of local lesions on cowpea half leaves inoculated with various combinations in the amount of the AMV infectious and mutant derived clones.

Mixture name	Amount of pCa17T per half leaf (μg)	Amount of pCa17TH per half leaf (μg)	Amount of pCa17TH(G) per half leaf (μg)	Amount of pCa17TH(T) per half leaf (μg)	Amount of each RNA 2-4 infectious clone per half leaf. (μg)	Number of local lesions. Mean ±s.d.
2W	2				2	4.0 ± 3.8
2W+2H	2	2			2	3.9 ± 1.9
2W+2G	2		2		2	2.0 ± 1.9
2W+2T	2			2	2	1.8 ± 0.4
2W	2				2	4.0 ± 3.8
2W+10H	2	10			2	8.0 ± 3.9
2W+10G	2		10		2	2.5 ± 1.1
2W+10T	2			10	2	1.0 ± 0.6

1)



2)

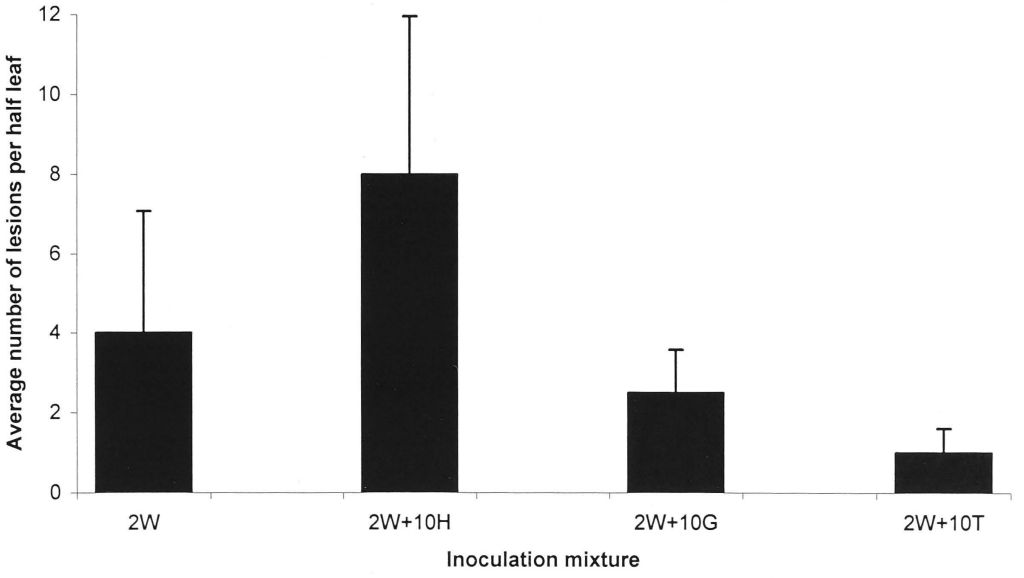


Figure 13: Mean number of lesions per half leaf (+s.d.) on cowpeas following inoculation with various combinations in the amount of the AMV infectious clones. All mixtures contained 2μg of each of RNA 2-4 infectious clones. The number(s) in the label for each inoculation mixture refers to the amount (μg) of the AMV RNA 1 infectious clone added. The letter 'W' refers to the unmodified AMV RNA 1 infectious clone pCa17T, whilst 'H' refers to pCa17TH, 'G' refers to pCa17TH(G) and 'T' to pCa17TH(T).

When the unmodified infectious clone, pCa17T, was co-inoculated with 2μg of pCa17TH (inoculation mixture A(H)) there was no change in the number of lesions compared to when 2μg of pCa17T alone was inoculated. However when 10μg of pCa17TH was co-inoculated with the unmodified infectious clone there was approximately a 100% increase in the number of lesions (inoculation mixture B(H)). This suggests that the pCa17TH infectious clone synthesizes additional functional AMV 1a protein or somehow increases the expression of the pCa17T infectious clone.

Two way analysis of variance statistical tests were carried out on the data. In the experiment where 2μg of the AMV RNA 1 derived plasmid clones were inoculated with 2ug of the AMV RNA 1 infectious clone plasmid there was no difference at the 5% level ($p=0.519$). When 10μg of the AMV RNA 1 derived plasmid clones were inoculated with 2μg of the AMV RNA 1 infectious clone there was no statistical difference between the different co-inoculation combinations at the 5% level, however there was marginal significant difference at the 5% level between the inoculation using the 'H' plasmid and the 'T' plasmid ($p=0.056$).

When pCa17T was co-inoculated with pCa17TH(G) or pCa17TH(T) there was approximately a 50% decrease in the number of lesions regardless whether 2μg or 10μg of the modified clones was inoculated (inoculation mixtures A(G), B(G), A(T) and B(T)). The most interesting comparison is that when 10μg of pCa17TH was inoculated with 2μg of pCa17T the number of lesions increased (mixture B(H)) but when 10μg of Ca17TH(G) or pCa17TH(T) was inoculated the number of lesions

decreased (mixtures B(G) and B(T), compared to when just 2μg of pCa17T was inoculated (mixture wt). This is strong evidence that the pCa17TH(G) and pCa17TH(T) clones produce AMV 1a protein defective in ATP binding and which can interfere with the replication of AMV.

4. DISCUSSION.

For the first time cowpeas have been used to quantitatively determine the infectivity of different combinations of the AMV infectious clones and their mutant derivatives to evaluate the effect of modifications to the AMV RNA 1 infectious clone. For the four experiments reported above, the inoculations were carried out at least twice using 4 replicates for each treatment. Bioassays on cowpeas can be notoriously variable due to variability between plants and inoculations. Because of this variability the typical standard deviation value for an experiment was relatively high compared to the mean however statistical significance was observed between a number of different treatments. None the less, the results from all of the experiments were consistent and reproducible between experiments.

The results reported in Section 3.1 (Figure 9) for the number of lesions from inoculating 2μg per half leaf of a mixture of all four unmodified infectious clones (pCa17T, pCa27T, pCa32T and pCa42T) is substantially higher than that for the same treatment in the results reported in Sections 3.2-3.4. This treatment was the positive control for each of the experiments reported. The experiments which

provided the results for Sections 3.2-3.4 were carried out during winter whilst that for Section 3.1 was carried out in early Autumn. Although the glasshouse was heated and cooled to maintain temperature, the cowpeas germinated and grew much slower and weaker during winter even when additional artificial light was provided to extend the period of daylight. An experiment (data not shown) which was undertaken in spring gave similar levels of infectivity as that in autumn. That being said, I consider the cowpea system of assessing the infectivity of infectious clone mixtures to be of potential value because the only alternate system uses tobacco in which AMV local lesion number is often difficult to quantify. I recommend increasing the number of replicates to reduce the size of the standard deviation relative to the mean so that statistically sound results can be obtained. It may also be useful to increase the concentration of the plasmid DNA for inoculating cowpeas when the plants are growing slower (data not shown) so as to increase the number of local lesions.

In all experiments when the pCa17TH(G) or pCa17TH(T) clones were inoculated in the absence of any other AMV RNA 1 construct no lesions were identified. This is strong evidence that the putative ATP binding motif identified in the AMV RNA 1 gene is essential for virus infection. This presumably suggests that both mutations stop the P-loop from undertaking ATP hydrolysis *in vivo*.

It is interesting that the co-inoculation of the mutant constructs with the unmodified AMV RNA 1 construct had reduced numbers of lesions compared to inoculations containing just the pCa17T construct or where the number was increased with the co-

inoculation of pCa17TH and pCa17T. This suggests that the constructs with the mutated ATP binding motif produce protein that interferes with the infectivity and presumably the replication of the virus whereas the pCa17TH construct produces functional AMV 1a protein. This is the first time that a defective viral protein has been shown to interfere with virus infectivity *in vivo.*

The lower infectivity of mixtures of the infectious clones containing increasing amounts of pCa17TH as the only AMV RNA 1 construct suggests that the insertion of the polylinker in the 3' untranslated region of the RNA 1 gene affects in some way viral infectivity. The 3'untranslated regions of each of the AMV RNAs 1,2 and 3 consist of a 3' terminal sequence of 145 nucleotides with high sequence similarity and an upstream sequence of 18 to 34 nucleotides that are unique for each RNA (van Rossum *et al*, 1997). Despite the occurrence of 22 individual nucleotide differences in the 145 nucleotide region between the different genomic RNAs, each can be folded similarly into five stem-loop structures (Fig 14.1, loops A-E) (van Rossum *et al,* 1997). The five stem loop structures are thought to be involved in the binding of the coat protein and the RNA replication complex formed by the 1a and 2a proteins (Reusken and Bol, 1996; van Rossum *et al*, 1997; Reusken *et al*, 1997). Mutations in the fifth stem-loop structure (Fig 14.1, loop E) from the 5' terminal end that disrupted base pairing reduced or abolished RNA replication (van Rossum *et al*, 1997). The 21 nucleotide polylinker inserted at the *DraIII* site to produce the pCa17TH plasmid is in the loop of this stem-loop structure and is predicted to cause a significant structural change (Fig14.2, loop E).

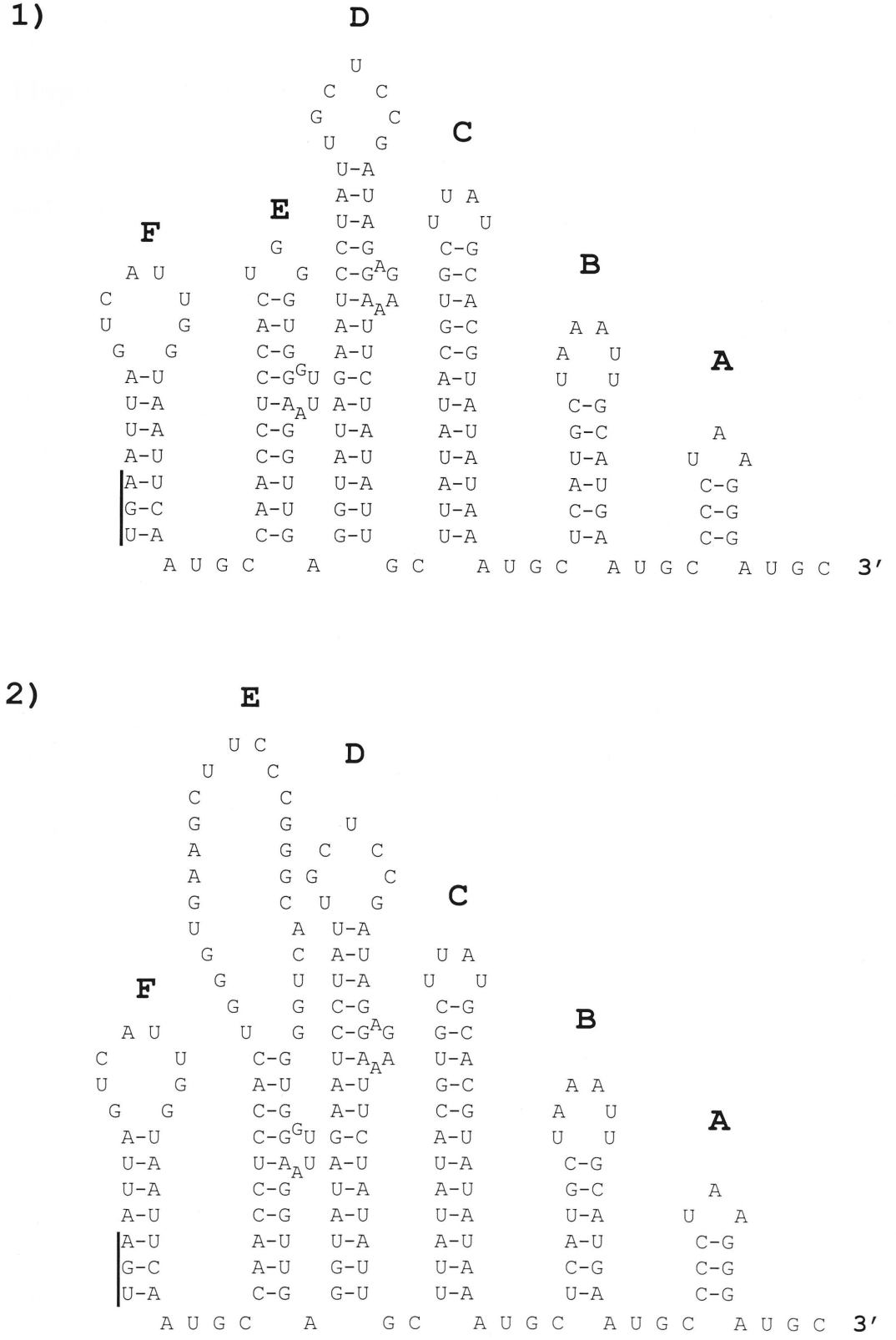


Figure 14: Schematic representation of the proposed secondary structure of the 3' untranslated region of AMV RNA 1 of the: 1) unmodified infectious clone, pCa17T; 2) infectious clone with the 21 nucleotide polylinker inserted in stem loop E, pCa17TH. The secondary structures are based on van Rossum *et al*, 1997. The black line indicates the stop codon. The stem loops labelled A to E are common to all of the genomic AMV RNAs. The stem loop labelled F varies in sequence and therefore in structure between the genomic RNAs.

I hypothesize that the modification to this stem-loop reduces the replication of the resultant AMV RNA 1 molecule through changing the parameters by which the replication complex and or coat protein bind, and thus to regulate the replication of the RNA concerned and the infectivity of the virus. However, in the presence of wild type AMV RNA 1 this modification has little negative effect on the replication of any AMV RNA 1 like molecule. Therefore the modified plasmids pCa17TH, pCa17TH(G) and pCa17TH(T) when inoculated in the presence of pCa17T are presumably transcribed and produce protein. Reverse transcriptase PCR and restriction enzyme analysis could be used to ascertain if the modified RNA 1 infectious clones are replicated along with the unmodified RNA 1 infectious clone.

On the basis of the revealing *in vivo* results I tried to obtain some AMV 1a protein by *in vitro* expression and affinity column purification so as to conduct some ATP binding assays on the wild type and mutated forms. Although it has been reported the proteins from a wide range of viruses has been expressed (Vlot *et al*, 2001), the only reported expression of an AMV protein is that of the coat protein in *E.coli* (Yusibov *et al*, 1996). The AMV RNA 1 gene was cloned into an *E.coli* expression vector pProEx-HTa (Promega) which has the *trc* promoter and *lac*I gene for inducible expression, a 6X histidine affinity tag for Ni^{2+} affinity column purification which can be cleaved from the protein by use of *Tobacco etch virus* protease (Figure 15). After repeated attempts in two different strains of *E.coli* (DH5α and DH10B), with different growth and gene induction conditions, with different selections of the same plasmid construct, 'wild type' or 'mutant T' protein could not be obtained from either the soluble or insoluble fractions. The only *Bromoviridae*

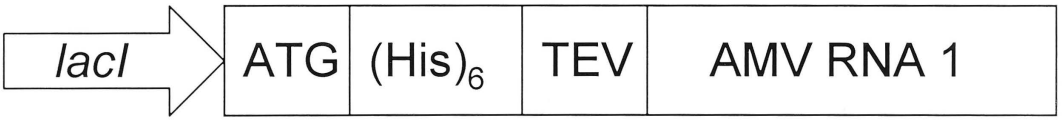


Figure 15: Schematic diagram of pProEX HT expression vector with AMV RNA 1 derived gene. The lacI repressor allows for regulation expression with IPTG, the $(His)_6$ allows for Ni^{2+} column affinity purification of the protein and the TEV (*Tobacco etch virus* protease cleavage site) permits the affinity tag to be removed following protein purification.

replicase protein to be expressed *in vitro* is that of *Brome mosaic virus* in yeast (Janda and Ahlquist, 1993). Thus an expression vector containing the AMV RNA 1 gene should be tried in yeast. Time did not permit further work to be carried out to try to purify *in vitro* expressed AMV 1a protein in yeast.

The key conclusions from the work described in this Chapter are that the putative ATP binding motif identified in the AMV 1a protein is essential for virus infection and that the mutations made to the motif make it dysfunctional. Based on these results I decided to express the wild type and mutant AMV RNA 1 gene constructs in plants and evaluate them for resistance to AMV infection in plants – this work is described in Chapter 3.

CHAPTER 3 DEVELOPMENT AND ANALYSIS OF TOBACCO AND WHITE CLOVER TRANSFORMED WITH AMV RNA 1 WILD TYPE AND ATP BINDING MOTIF MUTANTS FOR RESISTANCE TO AMV INFECTION.

1. INTRODUCTION.

In the previous Chapter, mutations to the putative ATP binding motif of the AMV RNA 1 infectious clone were made and evaluated. It was found that when the putative ATP binding motif was mutated the infectivity of the clones was negated. Furthermore, when DNA of the unmodified AMV RNA 1 infectious clone was co-inoculated with DNA of either of the mutated AMV RNA infectious clones along with a DNA mixture of the other required infectious clones (AMV RNAs 2-4) the level of infectivity was reduced. This suggests that the modified infectious clones, which are presumed to synthesize AMV 1a protein with defective ATP binding, are able to interfere with the infectivity of the wild type AMV RNA 1 infectious clone.

Given that the mutation of the putative ATP binding site in the AMV RNA 1 infectious clones negate the function of the protein synthesized, and that presumably this defective AMV 1a protein is able to compete with the wild type AMV 1a protein to form non-functional replication complexes with AMV 2a protein, the next step is to transform plants to express the mutant forms of AMV RNA 1 gene and to test for resistance to AMV infection. The two plant species chosen to evaluate this proposed mechanism of

virus resistance were tobacco, as a model plant system, and white clover as a commercial plant with high susceptibility and little natural resistance to AMV infection.

AMV isolate WC28 was used as the challenge virus as it gives rise in infected plants to clearly identifiable local lesions on tobacco and inter-vein clearing and necrosis on white clover. Furthermore, AMV isolate WC28 is a heterologous isolate to that of the source virus of the transgenes which is AMV strain 425 (Neeleman *et al*, 1993) and therefore is a better test for the durability of the new resistance mechanism.

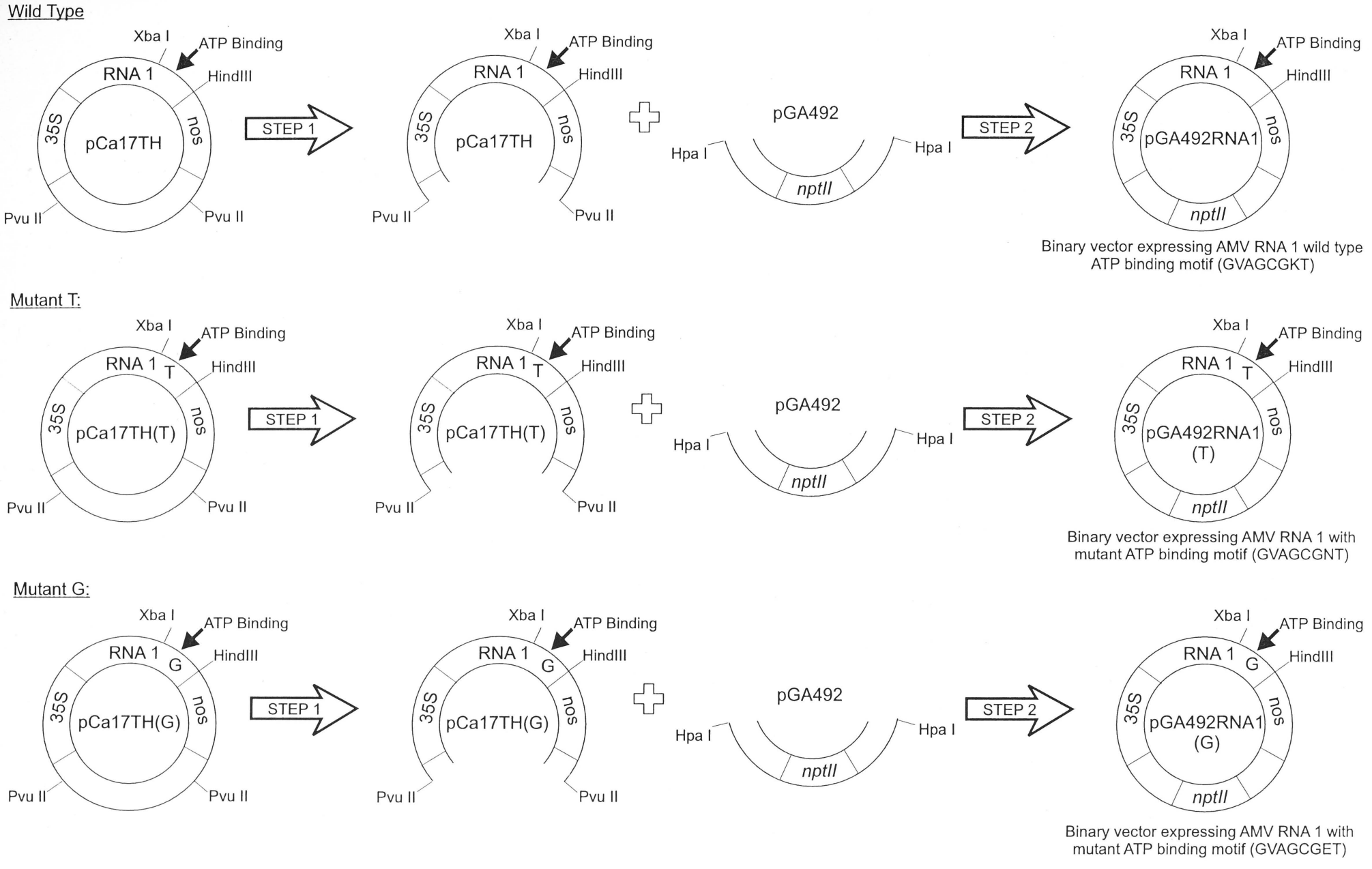
2. MATERIALS AND METHODS.

Materials and methods the same as that used in the previous Chapter are not re-described in this Section. All tissue culture specimens were grown in an enclosed room that was maintained at 26°C with 16 hours of artificial light.

2.1 Cloning strategy for the development of binary vectors containing the wild type and mutant ATP motif forms of the AMV RNA 1 gene.

The cloning strategy for the development of the binary vectors containing the wild type or mutant AMV RNA 1 gene will now be described (summarized in Figure 16). The plasmids pCa17TH (AMV RNA 1 infectious clone with a polylinker containing a *HindIII* restriction enzyme recognition sequence inserted at a *DraIII* site in the 3' untranslated region) and the plasmids pCa17TH(G) and pCa17TH(T) (plasmids the same as pCa17TH except that the DNA coding for the ATP binding motif has been

Figure 16: Diagram of the cloning strategy to produce pGA492 based binary vectors to express the AMV RNA 1 wild type and mutant genes.


Wild Type
Xba I
ATP Binding
RNA 1
HindIII
35S
pCa17TH
nos
Pvu II
Pvu II
STEP 1
pGA492
Hpa I
Hpa I
nptII
STEP 2
pGA492RNA1
Binary vector expressing AMV RNA 1 wild type ATP binding motif (GVAGCGKT)
Mutant T:
T
pCa17TH(T)
pGA492RNA1 (T)
Binary vector expressing AMV RNA 1 with mutant ATP binding motif (GVAGCGNT)
Mutant G:
G
pCa17TH(G)
pGA492RNA1 (G)
Binary vector expressing AMV RNA 1 with mutant ATP binding motif (GVAGCGET)

mutated) were digested separately with *PvuII* (step 1, Figure 16). The binary vector pGA492 (which has as the selectable marker for plant transformation the *nptII* gene with the 35S promoter and *nos* terminator to confer resistance to kanamycin) was digested with *HpaI*. The *PvuII* digested fragment of the pCa17TH and related mutant plasmids containing the RNA 1 gene was ligated with the *HpaI* fragment of pGA492 containing the left and right borders and the *nptII* gene (step 2, Figure 16). Since the restriction enzymes *PvuII* and *HpaI* cleave DNA to give a blunt end, the orientation of the ligated DNA was confirmed by a number of diagnostic restriction enzyme analysis so that the *nptII* and AMV RNA 1 genes were cloned in the same direction.

2.2 Transformation of *Agrobacterium.*

The triparental mating of *Agrobacterium tumefaciens* strain AGL1 with the pGA492 based binary vectors was carried out as described by Ditta *et al*, 1980. A glycerol stock of *Agrobacterium* was streaked out on MG/L-agar containing 20μg/ml rifampicin and grown at 28°C for 2 days. *E.coli* containing the binary plasmid vectors were streaked out on LB-agar containing 5μl/ml tetracycline and cultured overnight at 37°C. *E.coli* containing the helper plasmid RK2013 was also streaked out and grown overnight at 37°C on LB-agar containing 70μg/ml kanamycin. A wire loopful of each of the three bacterial cultures was mixed together and co-cultivated at 28°C for 2 days on MG/L-agar without any antibiotics. The mixed cultures were then streaked onto MG/L-agar containing 20μg/ml rifampicin and 5μg/ml tetracycline and cultured for 2 days at 28°C. Single colonies were then selected and re-streaked onto the same media. To confirm the transformation of the *Agrobacterium* had taken place and that no mutations had

occurred to the plasmid single colonies were selected and then grown in MG/L with antibiotics. Plasmid DNA was isolated from the *Agrobacterium* and transformed into *E.coli* by electroporation and then samples obtained for restriction enzyme analysis.

2.3 Transformation and regeneration of tobacco.

Transformation of tobacco cultivar W38 was carried out essentially as described by Horsh *et al* (1984). Young leaves from 4-8 week old tobacco plants in tissue culture were dissected in dH_2O into 1cm square segments and transferred to MS9-agar (MS media agar supplemented with 20g/L sucrose, 0.1g/L myo-inositol, 1mg/L benzyl amino purine and 0.5mg/L indole acetic acid) containing no antibiotics for 24 hours in the dark. Transformation of the leaf segments was carried out by floating the leaf segments in a culture of *Agrobacterium* (with an OD_{550nm} = 0.3) in MG/L with 5µg/ml tetracycline for several minutes before placing them adaxially onto freshly prepared MS9-agar containing 10mg/L kanamycin and 150mg/L Timentin™ (Beecham Research Laboratories; a 30:1 w/w mixture of sodium ticarcillin and potassium clavulanate) and incubated at 28°C for 10 days. Shoots growing from the segments were then transferred (only one shoot from any given callus to ensure independent transformants were obtained) about every 2 weeks to freshly prepared shooting media, MS9 containing 10mg/L kanamycin and 150mg/L Timentin™. When the shoots were at least 1cm in size they were transferred to freshly prepared rooting media, MSO (MS media agar supplemented with 30g/L sucrose, 0.1 g/L myo-inositol) containing 10mg/L kanamycin and 150mg/L Timentin™.

2.4 Transformation and regeneration of white clover.

The transformation and regeneration of white clover followed the protocol described by Larkin *et al*, 1996, with some small changes. White clover seed from a pure seed stock of the cultivar Haifa were surface-sterilized by soaking in 70% ethanol for 3 minutes, then 1.5% w/v available chlorine (bleach) solution for 40 minutes, then 70% ethanol for 3 minutes followed by 6 washes in dH_2O over a 60 minute period. The seeds were then imbibed overnight in dH_2O in the dark at 15°C. The seeds were carefully dissected to separate the imbibed cotyledons with a small segment of the tissue formerly attaching to the hypocotyl and epicotyl remaining with the cotyledons. The cotyledons were placed in MG broth (Garfinkle and Nester, 1980) containing a culture of *Agrobacterium* grown at 27° for 20-24 hours to a cell density of around $5X10^9$ cells/ml for about 1 hour. The cotyledons were washed with dH_2O then transferred to B5PB agar medium (Larkin *et al*, 1996)(containing no antibiotic) for 3 days at 24°C in light. After 3 days the cotyledons were washed several times with dH_2O and placed onto B5PB agar medium containing 300μg/ml Timentin™ and 25μg/ml of kanamycin. After 3 weeks, the cotyledons were subcultured onto the same media and again cultured for 3 weeks. Cotyledons with green shoot initials were transferred to the same media and cultured repeatedly every 3 weeks until several leaflets were formed. Only one leaflet was selected per cotyledon and was placed the rooting media RIB (basal salts and organics of L2, Philips and Collins 1984,) with 1.2μM of auxin (IBA) with 300μg/ml Timentin™ and 25μg/ml kanamycin.

When roots had been formed the plantlets were transferred to the glasshouse. Putative independent transformants were analysed for activity of the selectable marker *nptII* using a dot blot assay as described by McDonnell *et al*, 1987.

2.5 Plant RNA extraction.

The extraction of RNA from leaves followed with some modification the protocol of Higgins *et al*, 1976. Around 300mg of fresh tissue was thoroughly ground in liquid nitrogen. 600µl of NTES buffer (0.1M NaCl, 10mM Tris.HCl pH 8.0, 1mM EDTA and 1% w/v SDS) and 800µl of phenol/chloroform was added. The mixture was ground until thawed and immediately centrifuged for 5 minutes. The aqueous phase was collected and an equal volume of sterile 4M lithium chloride and 10mM EDTA was added and mixed thoroughly and then left on ice for at least 2 hours. The mixture was then centrifuged for 10 minutes. The supernatant was discarded and the pellet washed with sterile 70% ethanol and then allowed to air dry. The pellet was dissolved in 360µl of dH_2O and 40µl of 2M sodium acetate pH 5.8 and then centrifuged for 2 minutes. The supernatant was carefully collected and had 1ml of –20°C 100% ethanol added. The RNA was left to precipitate overnight at -20°C before centrifugation for 10 minutes. The pellet was washed with cold 70% ethanol and allowed to air dry. The pellet was dissolved in 20µl of dH_2O and the concentration of RNA estimated by measuring the absorbance at 260nm. Aliquots of RNA were precipitated with ethanol.

2.6 Preparation of randomly-primed radioactive probe.

The preparation of randomly-primed radioactive probe used the 'Ready-To-Go' labelling beads manufactured by Amersham-Pharmacia-Biotech (Cat. #27-9240-01) and followed the suggested protocol. Template DNA (50-200ng) in 25µl of H_2O was denatured by boiling for 3 minutes before being chilled on ice. The template DNA was added to the dCTP reaction beads which had been resuspended in 20µl of dH_2O. The mixture was incubated at room temperature for 5-10 minutes to which 5µl (50µCi) of α^{32}P-dCTP was added and mixed gently before incubation at 37°C for around 30 minutes. The unincorporated α^{32}P-dCTP was separated using a Micro-SPin G-50 Column manufactured by Amersham-Pharmacia-Biotech (Cat. #27-5335-01) following the suggested protocol.

2.7 Northern blot analysis.

To each lane of a 5% v/v formaldehyde 1.4% w/v agarose gel a 20µl sample made up of 10µl deionised formamide, 3.5µl deionised formaldehyde, 2µl 10X MOPS buffer, 1µl 10mg/ml ethidium bromide and 10µg of RNA in 3.5µl, was loaded with 5µl of RNA dye (8% w/v Ficoll 400, 0.02% w/v bromophenol blue and 0.04% w/v xylene cyanol). Each sample mixture was denatured by heating to 65°C for 5 minutes immediately before the RNA dye was added and the mixture loaded. The gel was run overnight and then blot transferred using 50mM NaOH to *Hybond-N*$^+$ membrane (Amersham-Pharmacia-Biotech) for about 3 hours. The membrane was then washed with 2XSSC buffer and was prehybridized for at least 2 hours and up to 48 hours in modified

southern buffer (0.2 w/v Ficoll, 0.2% w/v BSA, 0.2% w/v polyvinylpyrrolidone, 1% w/v SDS, 20μg/ml *E.coli* tRNA, 18μg/ml Herring Sperm DNA, 3XSSC and 0.05M HEPES pH 7.0). Hybridization was carried out for periods between 6 and 48 hours with labelled probe prepared as in Section 2.6 in modified southern buffer containing 10% w/v dextran sulphate. The blots were washed with 2XSSC at room temperature, then 2XSSC 0.1% SDS 0.1% Sodium pyrophosphate at 42°C and then 0.1% SSC 2XSSC 0.1% SDS 0.1% Sodium pyrophosphate at 42°C before the membrane was exposed to BioMax MS film (Kodak) at -80°C with a BioMax MS intensifying screen (Kodak). The film was processed according to the instructions of the manufacturer (Kodak).

2.8 RT-PCR reactions.

All RT-PCR reactions used the OneStep RT-PCR Kit manufactured by Qiagen (Cat.# 210212) and the suggested protocol was followed. For RT-PCR reactions to detect the *nptII* transcript, the 'Q' solution, which contains betaine, as provided by the manufacturer was used with the primers:

npt1177F (5' GCACAACAGACAATCGGCTGCTC 3') and

npt11922R (5' AGCACGAGGAAGGCGGTCAG 3').

The npt1177F primer is complementary to the *nptII* gene sequence 77 nucleotides from the start of the open reading frame. The npt11922R primer is complementary to the sequence 922 nucleotides from the start of the open reading frame. The primers are expected to produce a DNA fragment that is 845 base pairs. The temperature sequence used was; 50°C for 30 minutes, 95°C for 15 minutes, 94°C for 40 seconds, 50°C for 40

seconds, 72°C for 1 minute, with the last three steps repeated 35 cycles, followed by 72°C for 10 minutes.

For RT-PCR reactions to detect the AMV RNA 1 or related transcript, the primers used were:

amv1F (5' GAATGCTGACGCCCAATC 3') and

amv1R (5' CCATTTGTCCTTTGACTC 3').

The amv1F primer is complementary to the AMV RNA 1 sequence three nucleotides from the start of the open reading frame . The amv1R primer is complementary to the AMV RNA 1 sequence 1086 nucleotides from the start of the open reading frame. The primers are expected to produce a DNA fragment that is 1000 base pairs.

2.9 Mechanical virus inoculation of plants.

AMV isolate WC28 virus inoculum was obtained as discussed in Section 2.6 of Chapter 2. Tobacco plants were inoculated with 50µl per half leaf of the appropriately diluted (with 0.1M phosphate buffer pH 7.4) virus inoculum containing 1% w/v carborundum. The virus inoculum was applied to the first three seedling leaves (six half leaves) of tobacco plants that had been kept in the dark for a period of four to six hours and were then gently rubbed by hand across the leaves five times. After inoculation, the plants were washed with water.

White clover plants to be inoculated were kept in the dark overnight and for at least 4 hours the following day. 100µl of the virus inoculum with 1% carborundum was applied to each of three leaves (three leaflets each – 9 leaflets in total per plant) with

each leaflet being rubbed by hand five times. After inoculation, the plants were washed with water.

2.10 AMV ELISA assays.

Double antibody sandwich ELISA assays to estimate the level of AMV in the leaves of tobacco and white clover plants were as described by Clark and Adams, 1977.

Microtitre plates (Costar E.I.A./R.I.A. Flat Bottom High Binding microtitre plates, manufactured by Costar Corporation, USA) were coated by adding to each well 200µl of 0.5µg/ml of AMV specific IgG (Francki, S40, 87/4) in coating buffer (0.05M sodium carbonate, pH 9.6) and incubating at 37°C for 4 hours. The plate was emptied by a quick flick of the hand and rinsed once and then washed three times (3 minutes per wash) with PBS-Tween (10mM phosphate pH 7.4, 140mM NaCl, 3mM KCl and 0.05% Tween20) before the plates were drained and then sealed in a plastic bag and stored at -20°C.

Plant tissue was extracted in extraction buffer (100mM phosphate pH 7.4, 140mM NaCl, 30mM KCl, 0.05% Tween20 and 2% PVP) using a sap extractor at a ratio of tissue mass:volume of buffer ratio of 1:5 (w/v). Duplicate aliquots of 200µl were applied to wells of the plates and incubated overnight at 4°C. The plates were then rinsed once and then washed 3 times as above with PBS-Tween. Then 200µl of AMV specific IgG alkaline phosphatase conjugate (Francki, S40, 87/4) diluted to 0.25µg/ml in conjugate buffer was added to each well with the plates then incubated at 37°C for 4

hours. The plates were then rinsed and washed with PBS-Tween as described above. The plates were then drained before 250µl of freshly prepared substrate solution (0.6mg/ml 2-nitrophenyl phosphate in 9.7% v/v diethanolamine pH 9.8) was added to each well and then the plates were incubated at room temperature for 1 hour. The relative level of hydrolysed enzyme substrate was determined by measuring the absorbance at 405nm using a Multiskan Plus microtitre plate reader manufactured by LabSystems.

2.11 Visual scoring of symptoms on virus inoculated tobacco plants.

Tobacco plants were assessed for the number of lesions on each inoculated leaf. A score from 0 to 5 was given for each leaf on a plant. Score 0 was given when no lesions could be observed, score 1 when 1 to 20 lesions were present, score 2 when 21 to 40 lesions were present, score 3 for 41 to 80 lesions, score 4 for 81 to 160 lesions and a score of 5 if more than 160 lesions were present.

3. RESULTS.

3.1 Evaluation of transgenic tobacco containing the wild type and mutant ATP motif forms of the AMV RNA 1 gene for resistance to AMV infection.

Twelve independent putative transformed tobacco plants were selected for each binary vector – pGA492RNA1 (wild type – coded as 'W'), pGA492RNA1(G) (Mutant G

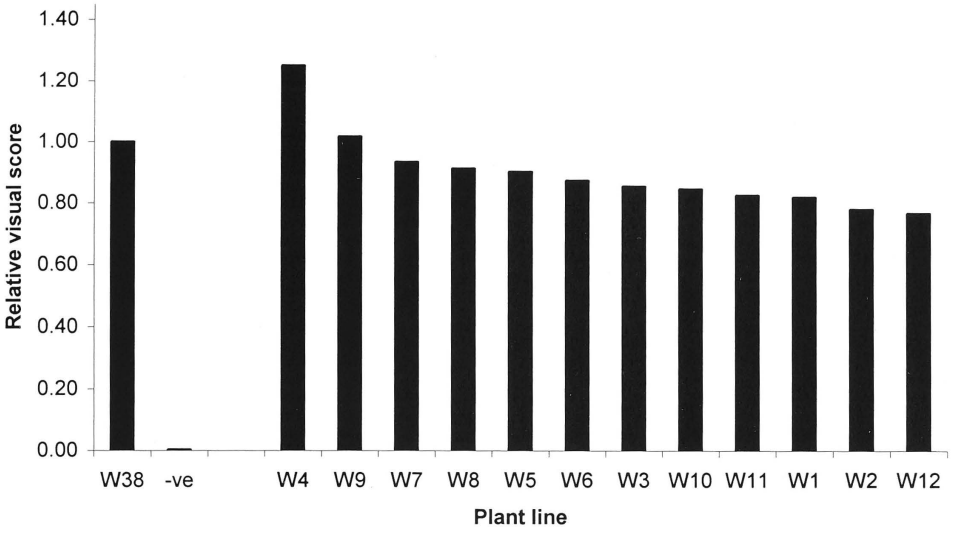
coded as 'G') and pGA492RNA1(T) (Mutant T coded as 'T') – and were confirmed to be transgenic by PCR analysis for the presence of the *nptII* gene. Each transgenic line had at least 16 and up to 24 vegetatively propagated clones were made, including the untransformed line 'W38' which was used as the negative control.

Eight clones were selected for each transgenic that had their first three seedling leaves fully formed and which were all similar between lines for the stage of growth and size. Three were inoculated with 1:50 w/v dilution of AMV isolate WC28 virus inocula and three with 1:100 w/v dilution of the same inocula and two were not inoculated as the negative controls with one for each inoculation. It was not always possible to inoculate all of the transgenic lines at a single time. However, all inoculations were carried out with three replicate plants inoculated and one not inoculated for each line along with 'W38' plants as the internal control reference.

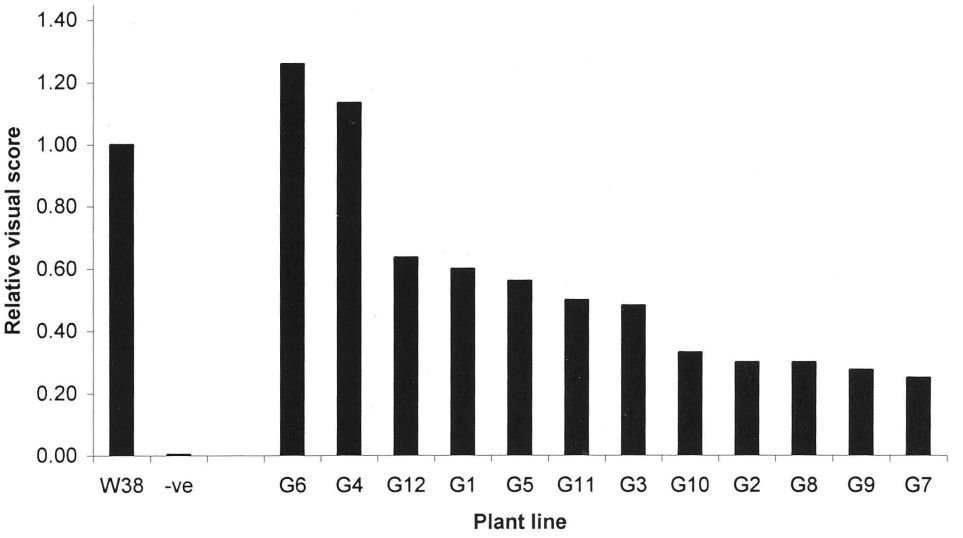
The visual score of symptom severity (number of local lesions) on each inoculated leaf of each plant was assessed six to eight days after inoculation. The results for the three inoculated leaves were combined with those of the other plants of the same transgenic line. Differences were observed between lines transformed with the 'W' construct compared to the lines transformed with the 'G' and 'T' constructs (Figure 17). All of the 'W' lines had a similar visual score to the untransformed 'W38' internal control reference plants (Figure 17.1). The 'G' and 'T' lines had a range in the visual score with a number of plant lines having a low score with of less than 50% of the 'W38' control and others being similar to that of the 'W38' line (Figures 17.2 and 17.3).

Figure 17: Relative visual score six days after inoculation of transgenic tobacco plants containing the: 1) wild type AMV RNA 1 gene; 2) Mutant T AMV RNA 1 gene and; 3) Mutant G AMV RNA 1 gene. All values were calculated relative to the value obtained from the inoculated untransformed control (WC38). The negative control (-ve) was W38 plants that were not inoculated with virus inocula. Three leaves on each of three plants were inoculated in each experiment, with the experiment repeated twice. The results given are an average of the three leaves from the six plants.

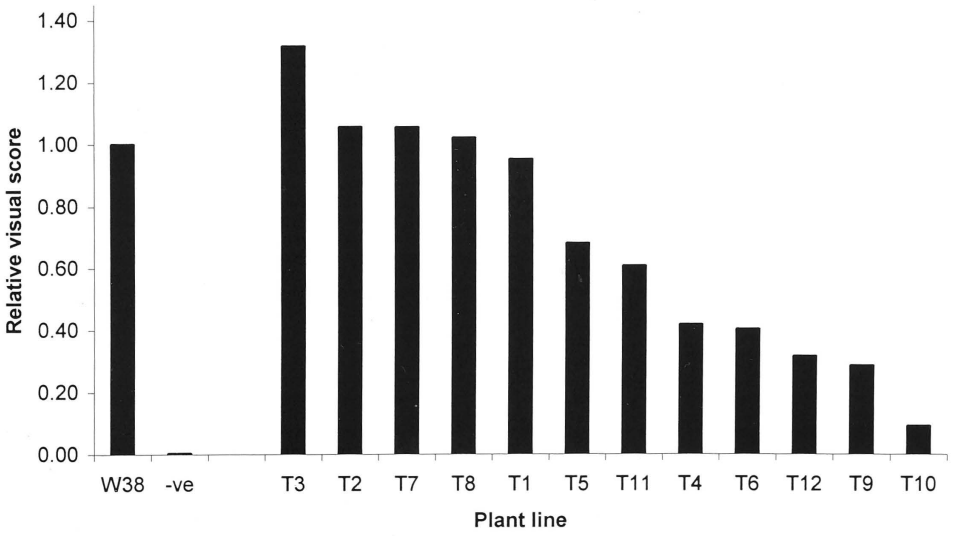
1)


1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Relative visual score
W38
-ve
W4
W9
W7
W8
W5
W6
W3
W10
W11
W1
W2
W12
Plant line

2)


1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Relative visual score
W38
-ve
G6
G4
G12
G1
G5
G11
G3
G10
G2
G8
G9
G7
Plant line

3)


1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Relative visual score
W38
-ve
T3
T2
T7
T8
T1
T5
T11
T4
T6
T12
T9
T10
Plant line

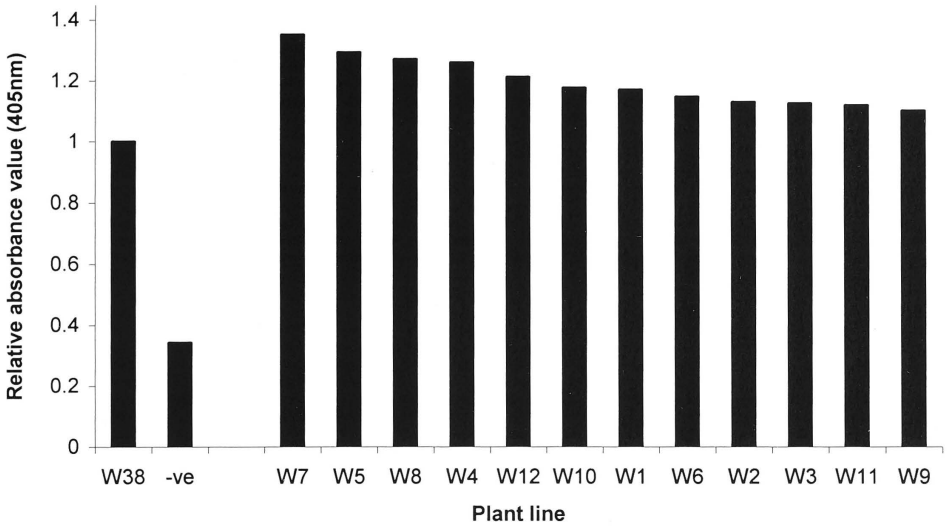
ELISA assays were conducted on 1:1000 v/v diluted sap extracted from leaf discs taken from the three inoculated leaves of each plant six to eight days after inoculation. As for the visual score, the results for the three leaves were combined with that of the other plants of the same transgenic line (Figure 18). The 'W' lines generally had a higher ELISA reading than the 'W38' internal control reference plants (Figure 18.1). In contrast, the 'G' and 'T' lines had a range of ELISA readings with some lines being similar to the 'W38' control plants but with others similar to that of the '-ve' (un-inoculated) control plants (Figures 18.2 and 18.3).

When the visual score for each of the twelve transgenic lines is compared with the corresponding ELISA result, the 'W' series plants have a similar value for both assessments as seen in the cluster in the top right hand corner of Figure 19. Conversely, the 'G' and 'T' series plants show a range of phenotypes varying from those having visual scores and ELISA values similar to that of the 'W' series, to that where both the visual score and ELISA values are considerably lower than the 'W' series (Figure 19). It is clear that the 'G' and 'T' series plants have attenuated symptoms of virus infection in contrast with the 'W' series plants which have the same symptoms of infection as the untransformed plants.

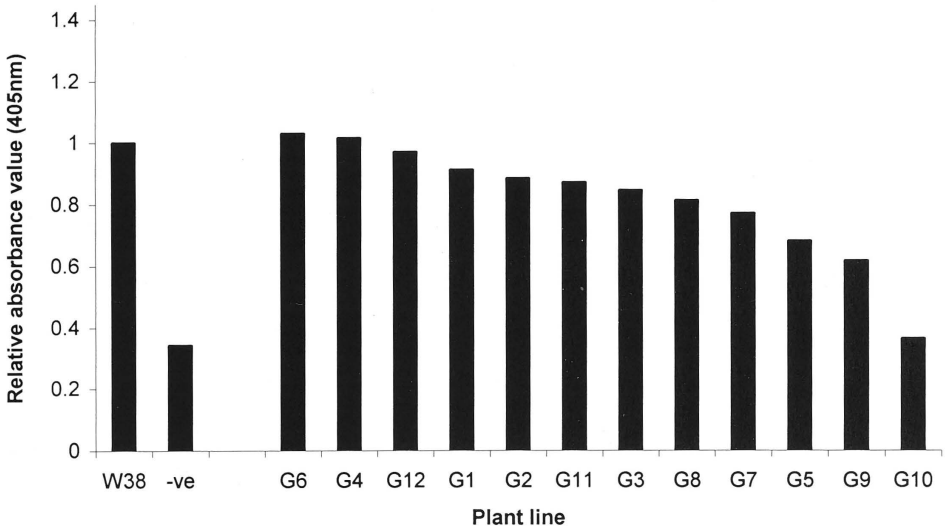
Plant lines showing a range of resistance to AMV infection were selected and replicate plants were inoculated as before to verify the previous experiment. The mean visual score and ELISA values were, in general, consistent with that observed previously (Figures 20 and 21). Also consistent with previous observations was that the first seedling leaf showed fewer symptoms and contained less virus following inoculation

Figure 18: Relative absorbance value (405nm) of ELISA assays on leaf discs taken 6 days after inoculation with AMV isolate WC28 inocula from transgenic tobacco containing the: 1) wild type AMV RNA 1 gene; 2) Mutant T AMV RNA 1 gene and; 3) Mutant G AMV RNA 1 gene. All values were calculated relative to the value obtained from the inoculated untransformed control (W38). The negative control (-ve) was W38 plants that were not inoculated with virus inocula. Three leaves on each of three plants were inoculated in each experiment, with the experiment repeated twice. The results given are an average of the three leaves from the six plants.

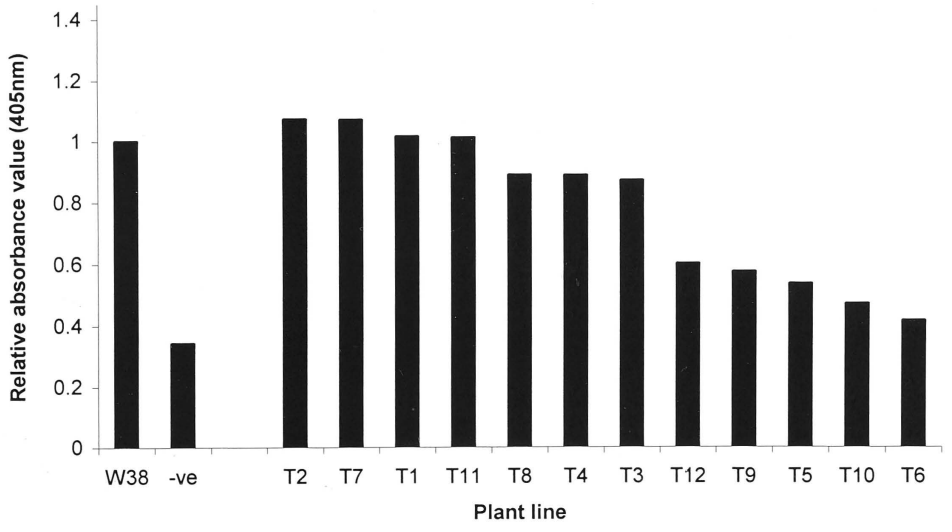
1)


Relative absorbance value (405nm)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
W38 -ve W7 W5 W8 W4 W12 W10 W1 W6 W2 W3 W11 W9
Plant line

2)


Relative absorbance value (405nm)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
W38 -ve G6 G4 G12 G1 G2 G11 G3 G8 G7 G5 G9 G10
Plant line

3)


Relative absorbance value (405nm)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
W38 -ve T2 T7 T1 T11 T8 T4 T3 T12 T9 T5 T10 T6
Plant line

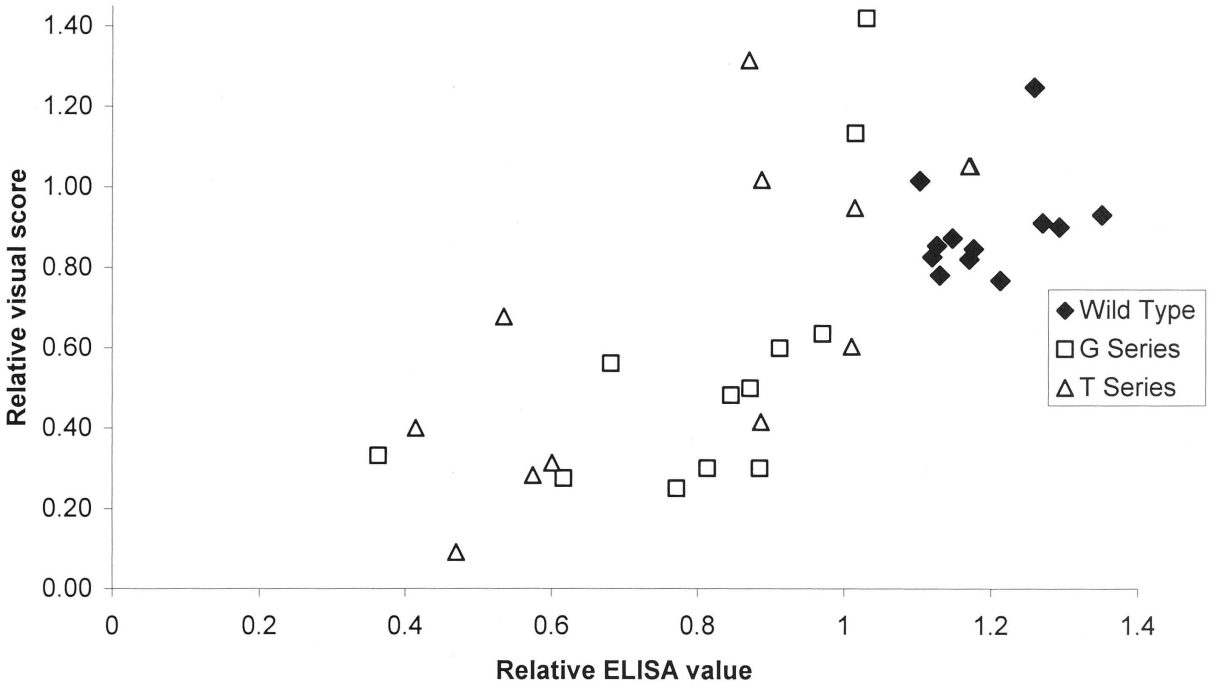
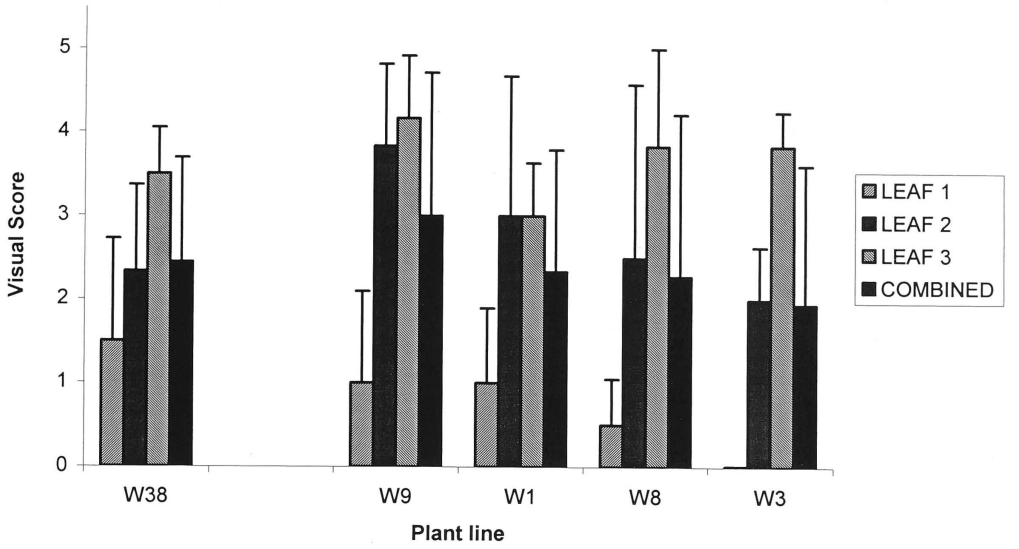


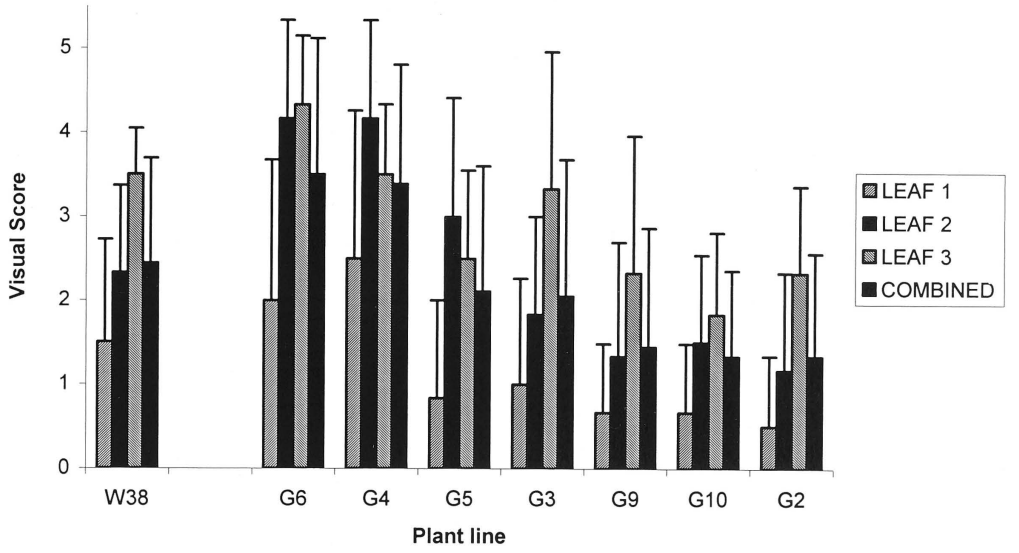
Figure 19: Graph of the relative visual score vs the relative ELISA value for transgenic tobacco expressing the three different AMV RNA 1 constructs.

Figure 20: Visual score for leaf 1, leaf 2 and leaf 3 eight days post inoculation with AMV isolate WC28 inocula at a 1:100 w/v dilution for transgenic tobacco containing the 1) wild type AMV RNA 1 gene; 2) Mutant G AMV RNA 1 gene and; 3) Mutant T AMV RNA 1 gene. Three replicate plants were inoculated with the mean and standard deviation calculated and shown. Three positive control plants were inoculated (WC38). For each line one plant was not inoculated and was otherwise treated the same as a negative control – none of these plants had any symptoms of virus infection. Plants in each transgenic line are ordered left to right in decreasing mean value.

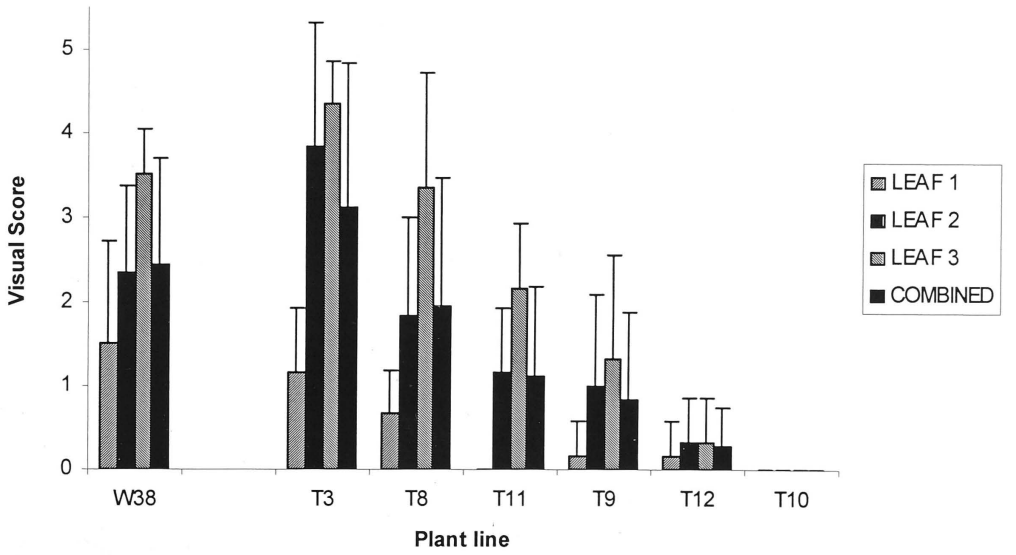
1)


Visual Score
0
1
2
3
4
5
W38
W9
W1
W8
W3
Plant line
LEAF 1
LEAF 2
LEAF 3
COMBINED

2)


Visual Score
0
1
2
3
4
5
W38
G6
G4
G5
G3
G9
G10
G2
Plant line
LEAF 1
LEAF 2
LEAF 3
COMBINED

3)


Visual Score
0
1
2
3
4
5
W38
T3
T8
T11
T9
T12
T10
Plant line
LEAF 1
LEAF 2
LEAF 3
COMBINED

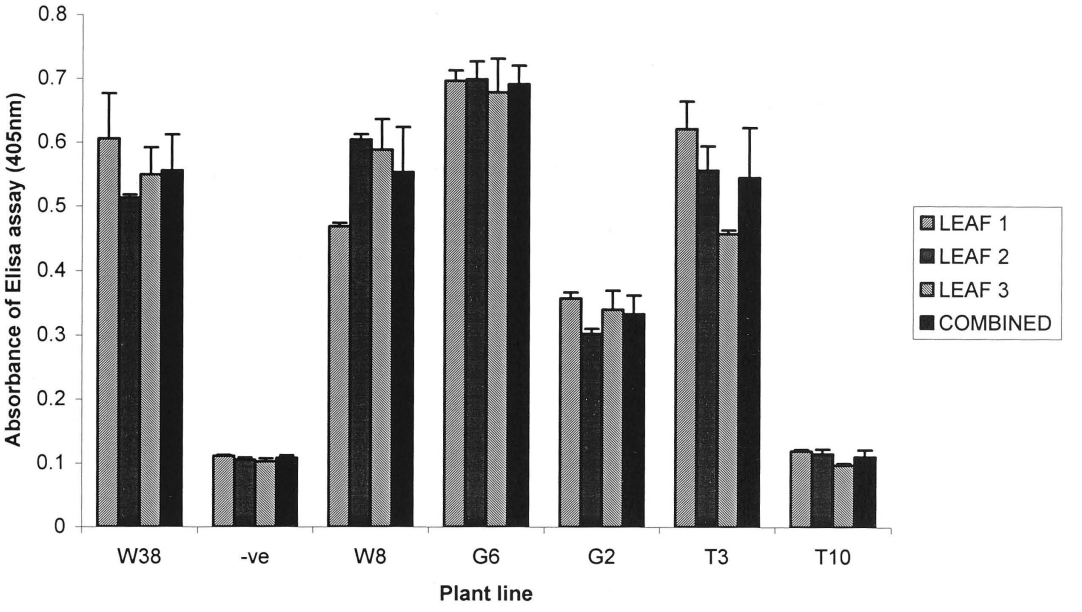


Figure 21: Mean absorbance values (+s.d.) of ELISA assays of sap extracted in a 1:1000 dilution of leaf discs taken from the 1st, 2nd and 3rd inoculated leaves of transgenic tobacco. Plants were inoculated with 1:50 w/v dilution of AMV isolate WC28 inoculum with duplicate leaf discs taken from each leaf. The 'W38' line is untransformed tobacco strain WC38, the '-ve' line is the same tobacco that was not inoculated. The W8 line was transformed with a construct containing the wild type AMV RNA 1 gene, plants G6 and G2 are independent transformants containing the Mutant G AMV RNA 1 gene, and plants T3 and T10 are independent transformants containing the Mutant T AMV RNA 1 gene.

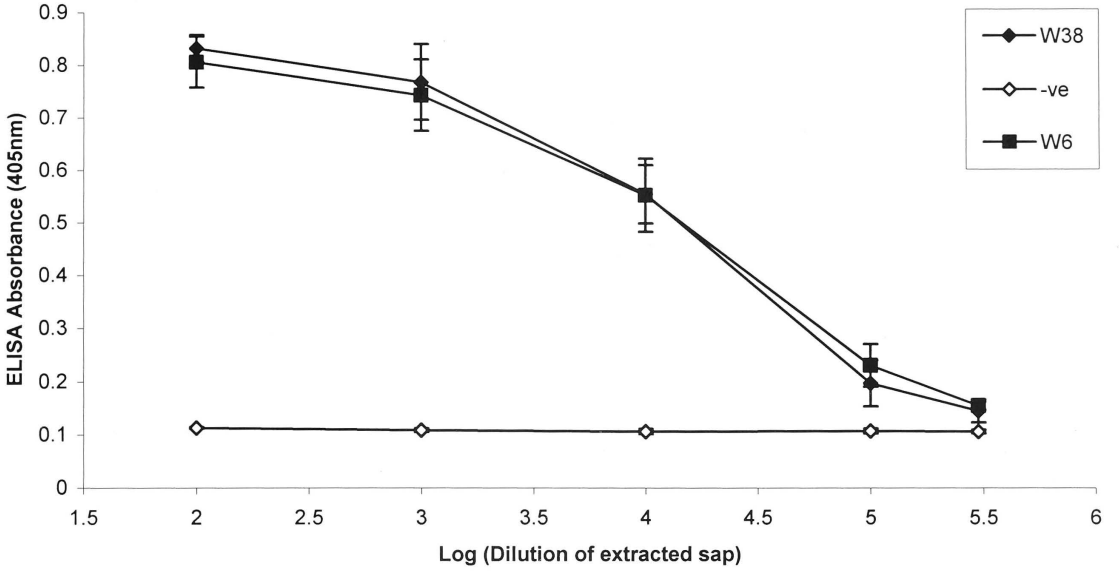
than the second or third leaves (Figures 20 and 21). It was also found that the use of the mean value for all three leaves showed good correlation with any individual leaf in relative terms.

To investigate further the resistance response of select plants, another inoculation was undertaken. After inoculation, serial dilutions were made of the extracted sap from the leaf strips of the inoculated leaves for ELISA analysis. The results are given in Figure 22. The W6 had a similar level of virus as to the control W38 (Figure 22.1). The G6 had a slightly lower level than the W38 line whilst the G9 line had a somewhat lower level (Figure 22.2). The T3 was very similar to the W38 whilst the T10 line had very little virus detectable, barely above the '-ve' control (Figure 22.3). A good indication of the relative levels of virus in each line can be observed by looking at the comparative dilution level required using linear regression analysis for each line to produce a given ELISA absorbance value within the linear part of the dilution series (Figure 23). From this we can see that lines G9 and T10 have low levels of virus whilst other lines of the same respective series, G6 and T3, have much higher levels

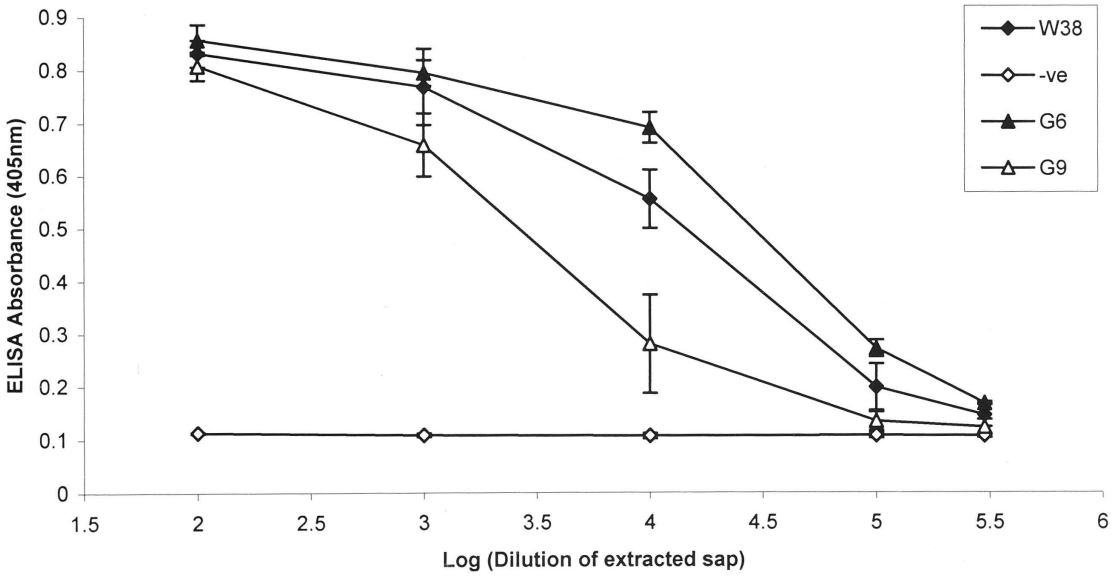
So far I have discussed only results relating to the inoculated leaves. It was evident that plants showing lower levels of virus infectivity in the inoculated leaves had a delay or a decrease in the symptoms of virus infection in the first systemic leaf. It is difficult to visually score the systemic leaves of AMV infected plants because the primary symptom is 'clearing' of the veins followed several days later by localised chlorosis. Therefore, serial dilution ELISA assays were conducted on leaf strips of the first systemic leaves 10 days after inoculation on the same plants for which the serial

Figure 22: Mean absorbance values (± s.d.) of ELISA assays of sap extracted from the inoculated leaves of transgenic tobacco containing the 1) wild type AMV RNA 1 gene; 2) Mutant G AMV RNA 1 gene and; 3) Mutant T AMV RNA 1 gene. Sap was extracted from leaf discs taken from the 1st, 2nd and 3rd inoculated leaves of three replicate plants for each line six days after inoculation with 1:100 w/v dilution of AMV isolate WC28 virus. The sap was diluted at 1:100. 1:1000, 1:10000, 1:100000 and 1:300000 v/v. The positive control (W38) is inoculated untransformed tobacco strain W38 with the negative control being the same strain but which was not inoculated.

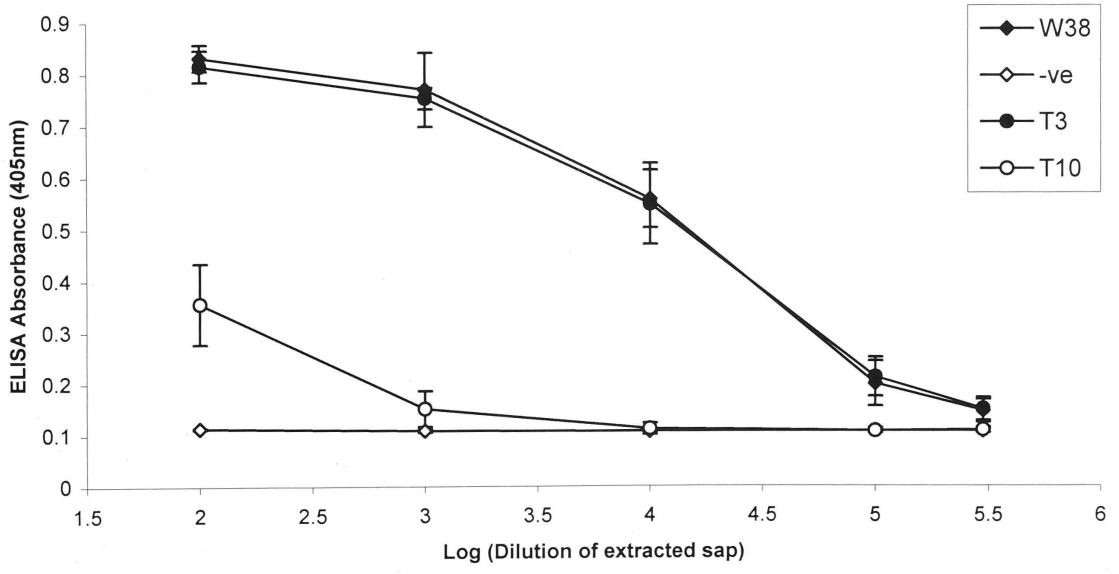
1)


ELISA Absorbance (405nm)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1.5
2
2.5
3
3.5
4
4.5
5
5.5
6
Log (Dilution of extracted sap)
W38
-ve
W6

2)


ELISA Absorbance (405nm)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1.5
2
2.5
3
3.5
4
4.5
5
5.5
6
Log (Dilution of extracted sap)
W38
-ve
G6
G9

3)


ELISA Absorbance (405nm)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1.5
2
2.5
3
3.5
4
4.5
5
5.5
6
Log (Dilution of extracted sap)
W38
-ve
T3
T10

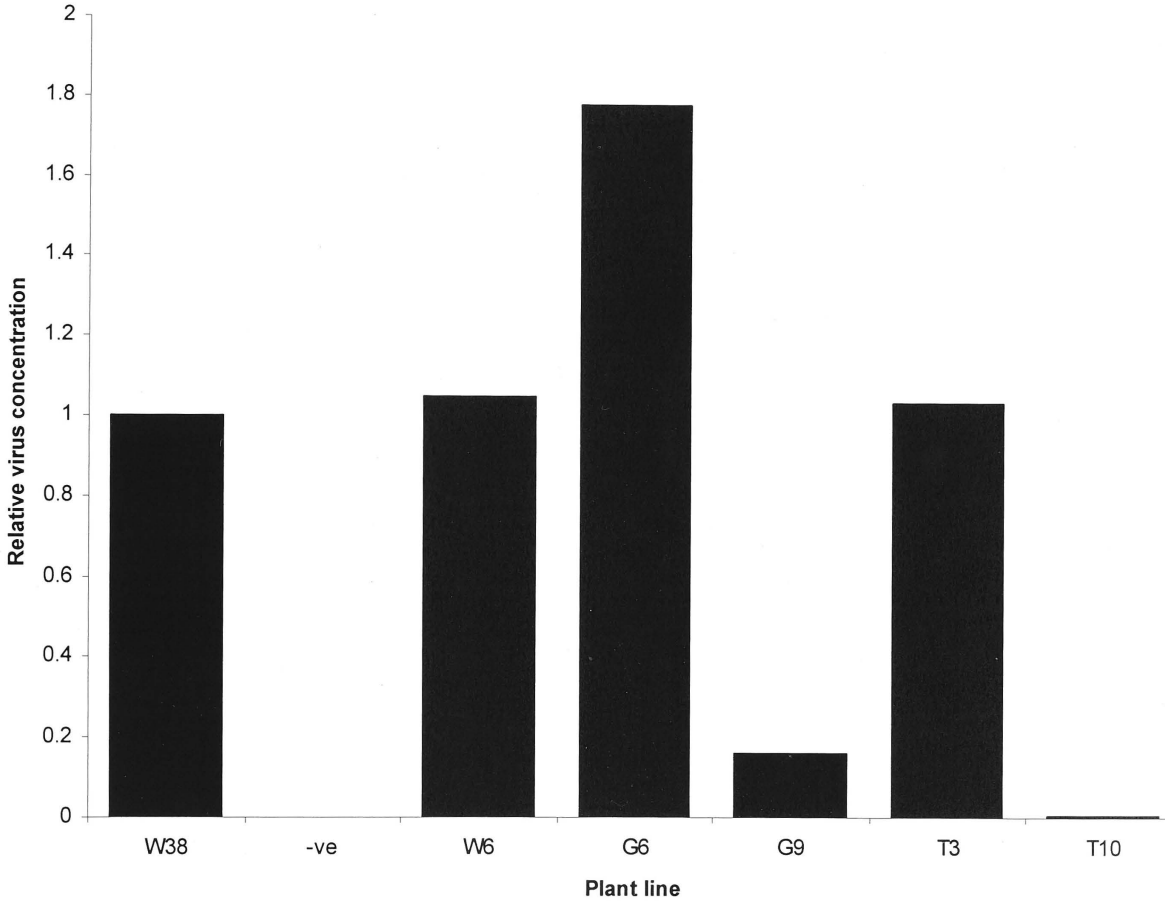


Figure 23: Relative virus concentration in tobacco plants inoculated with AMV isolate WC28 viral inocula. Sap was extracted from leaf discs taken from the 1st, 2nd and 3rd inoculated leaves of three replicate plants for each line six days after inoculation with 1:100 w/v dilution of the AMV isolate WC28 viral inocula. The sap was diluted at 1:100, 1:1000, 1:10000, 1:100000 and 1:300000 v/v. The relative virus concentration was determined using linear regression analysis for each tobacco plant line using the point values in the linear part of the ELISA dilution curve for the dilutions 1:1000, 1:10000 and 1:100000 and an ELISA absorbance value of 0.3.

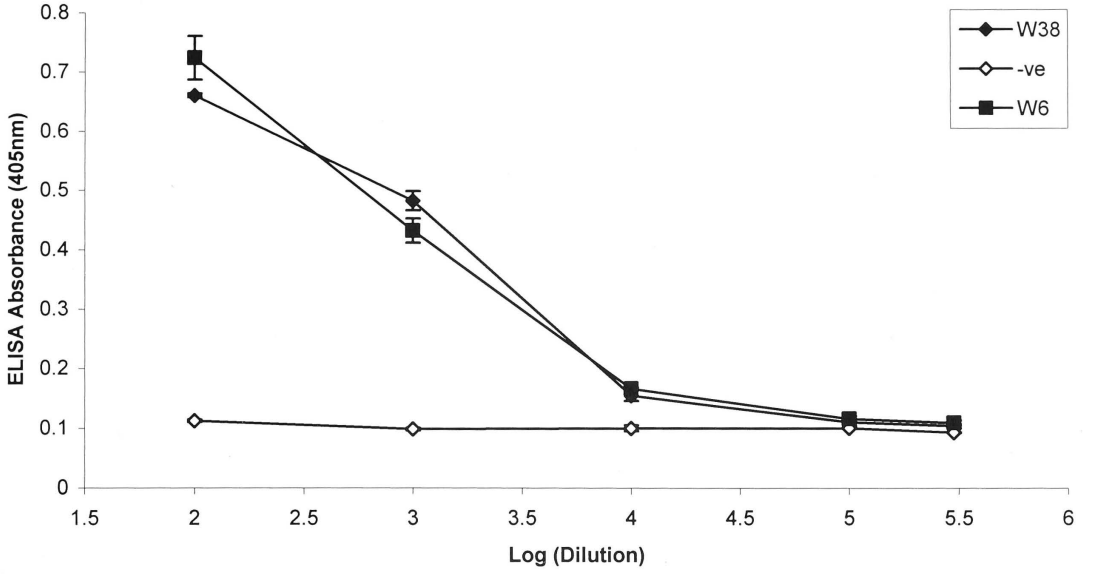
dilution ELISA assays were conducted. The results are given in Figure 24 and correlate with that of the inoculated leaves (Figures 22 and 23).

All transgenic tobacco lines containing the 'W' construct had similar symptoms, the number of local lesions and the degree of necrosis, of virus infection as the untransformed control line 'W38' at the plant (Figures 25A and 25B) and inoculated leaf level (Figures 26A and 26B). In contrast, transgenic tobacco lines containing the 'G' and 'T' lines showed a range of symptoms of virus infection with a few lines (G4, G6, T3, T2, T7 and T8) being similar to that of 'W38' and the 'W' lines through to the remaining majority of lines showing very moderated symptoms at the plant level (Figure 25) and at the inoculated leaf level (Figure 26).

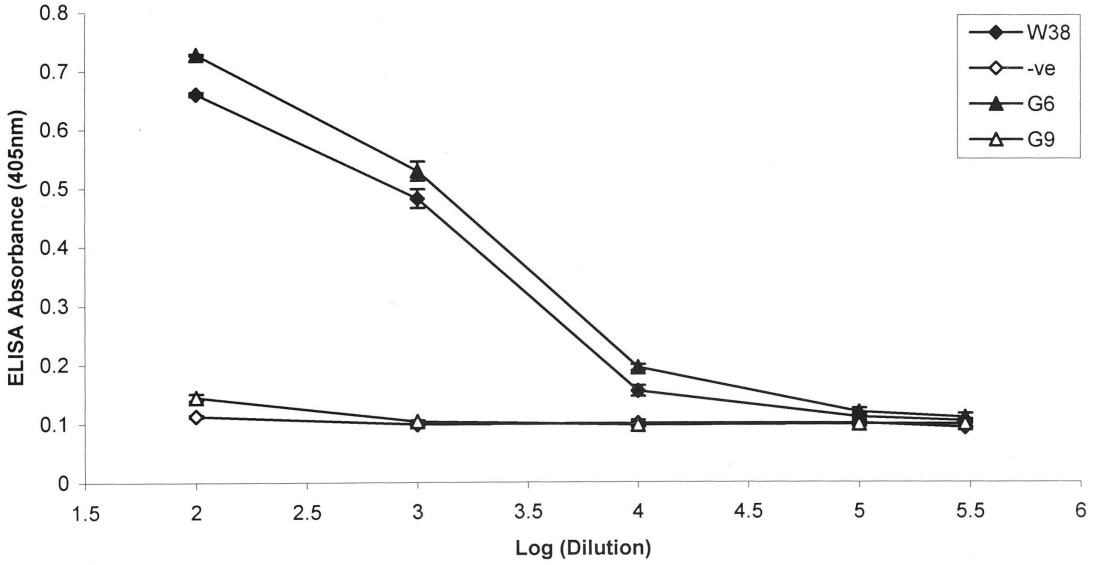
A most striking observation is that in all of the 'G' and 'T' lines (except for G6, G4 and T3) the necrotic local lesions on the inoculated leaves did not have the characteristic ring formation (Figures 27C and 27D) that is typical of AMV infection and which was observed on all of the 'W' lines and the 'W38' control line (Figures 27A and 27B). It was observed that in some replicate plants of the G6, G4 and T3 lines there was some attenuation of the ring formation in the necrotic local lesion. All of the un-inoculated control and inoculated tobacco plants were kept for around eight weeks after inoculation to see if symptom recovery could be observed. No recovery beyond the normal symptom attenuation was observed as the plants matured.

Figure 24: Mean absorbance values (± s.d.) of ELISA on sap extracted from the first systemic leaf of transgenic tobacco containing the: 1) wild type AMV RNA 1 gene; 2) Mutant G AMV RNA 1 gene and; 3) Mutant T AMV RNA 1 gene. Sap was extracted from the leaf discs of the 1st systemic leaf of three replicate plants for each line six days after inoculation with 1:100 w/v dilution of AMV isolate WC28 virus. The sap was diluted at 1:100, 1:1000, 1:10000, 1:100000 and 1:300000 v/v. The positive control (W38) is inoculated untransformed tobacco strain W38 with the negative control being the same strain but which was not inoculated.

1)


W38
-ve
W6
ELISA Absorbance (405nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1.5
2
2.5
3
3.5
4
4.5
5
5.5
6
Log (Dilution)

2)


W38
-ve
G6
G9
ELISA Absorbance (405nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1.5
2
2.5
3
3.5
4
4.5
5
5.5
6
Log (Dilution)

3)

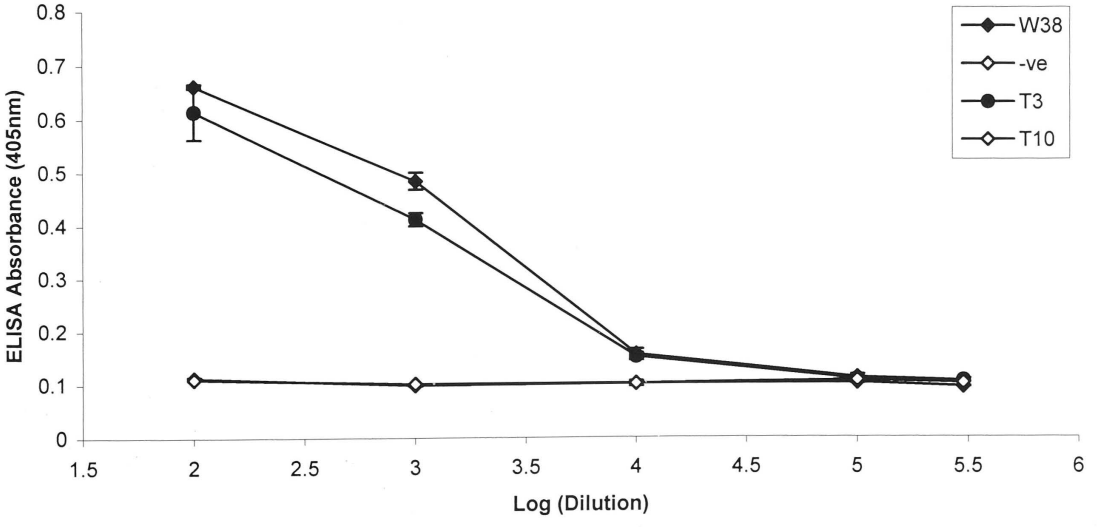

W38
-ve
T3
T10
ELISA Absorbance (405nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1.5
2
2.5
3
3.5
4
4.5
5
5.5
6
Log (Dilution)

Figure 25: Tobacco plants inoculated with a 1:50 w/v dilution of AMV isolate WC28 virus inocula onto the first, second and third seedling leaves eight days after inoculation; A) untransformed control line 'W38'; B) transformed line 'W6'; C) transformed line 'G9 and; D) transformed line 'T12'.

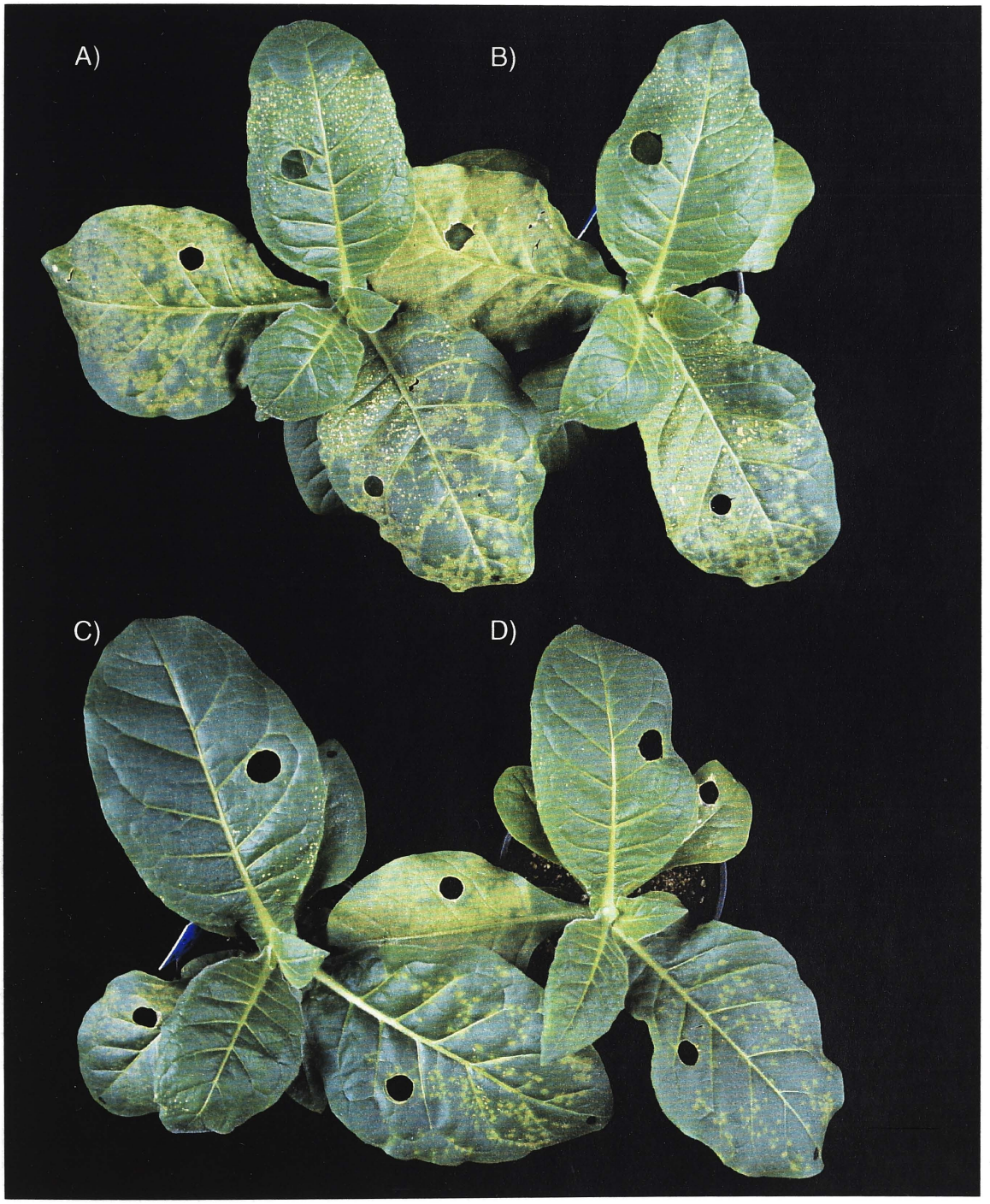

A)
B)
C)
D)

Figure 26: Inoculated leaves of tobacco plants inoculated with a 1:50 w/v dilution of AMV isolate WC28 virus inocula onto the first, second and third seedling leaves eight days after inoculation; A) untransformed control line 'W38';
B) transformed line 'W6'; C) transformed line 'G9 and;
D) transformed line 'T12'.
CR: Chlorotic rings, typical of AMV infection
NS: Necrotic spot, typical of AMV infection
LL: local lesion with reduced or absent chlorotic rings

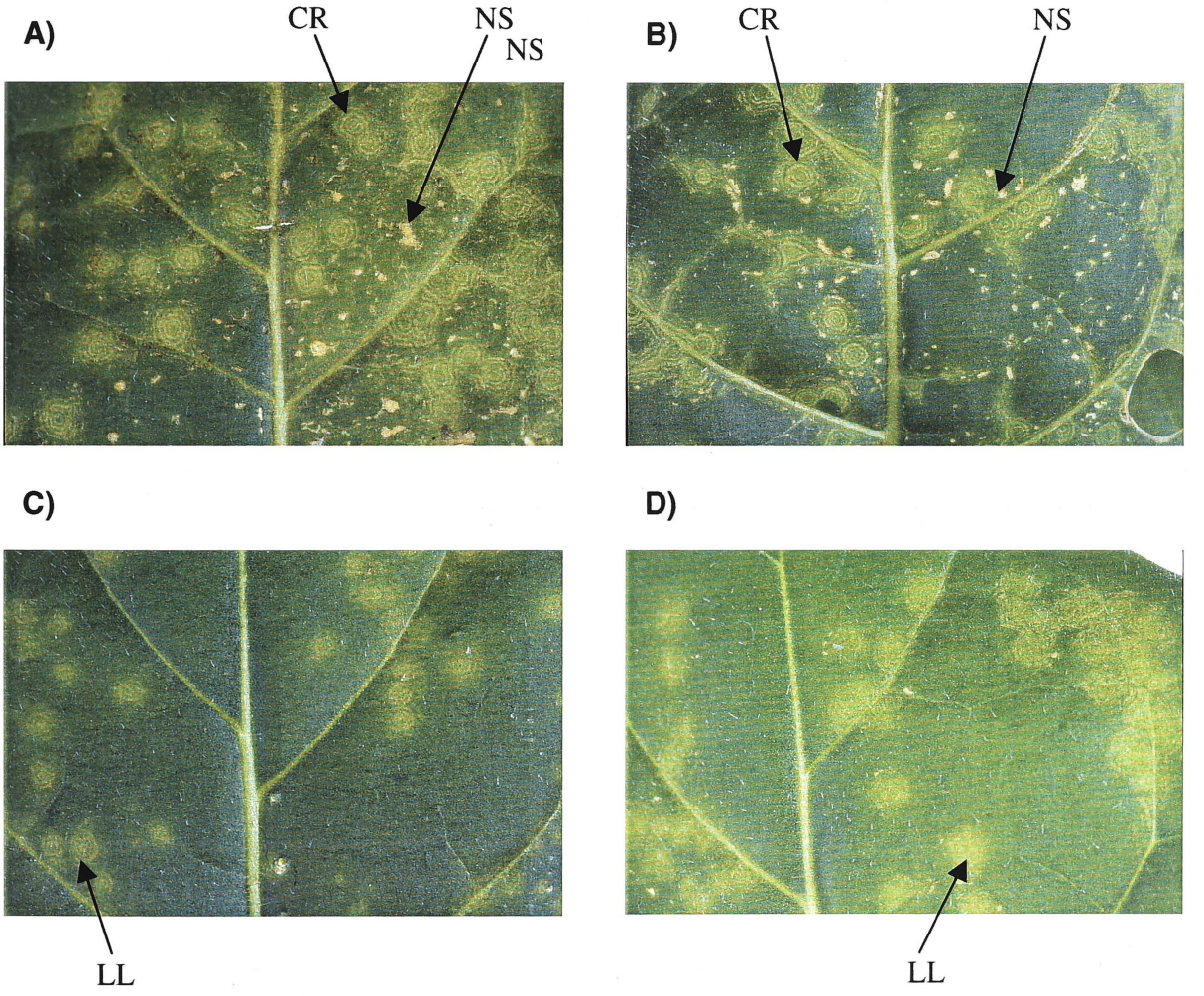
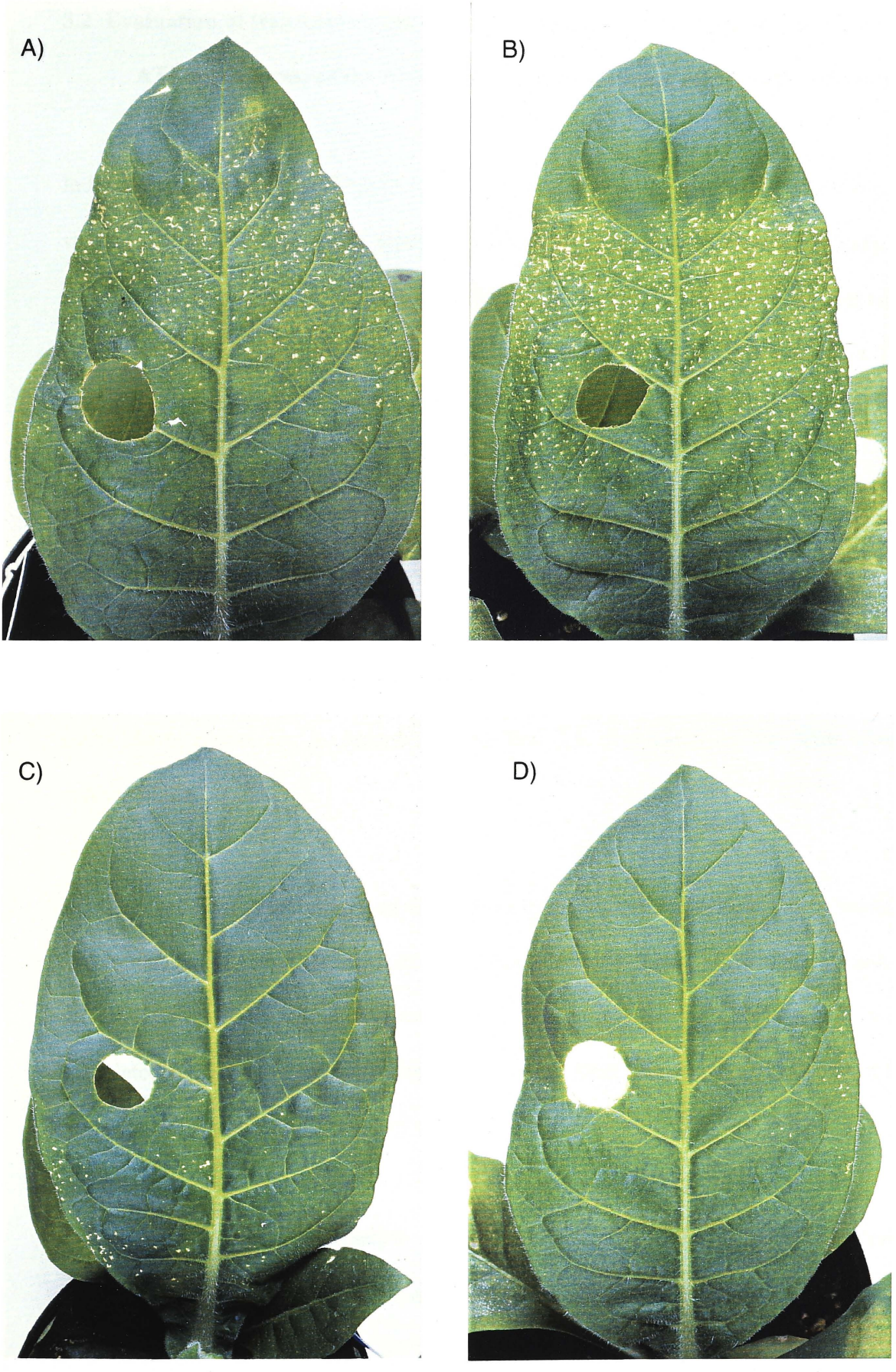

A)
CR
NS
NS
B)
CR
NS
C)
LL
D)
LL

Figure 27: Local lesions on the first systemic leaves of tobacco plants inoculated with a 1:50 w/v dilution of AMV isolate WC28 virus inocula onto the first, second and third seedling leaves eight days after inoculation; A) untransformed control line 'W38'; B) transformed line 'W6'; C) transformed line 'G9 and; D) transformed line 'T12'.


A)
B)
C)
D)

3.2 Evaluation of transgenic white clover containing the wild type and mutant ATP motif forms of the AMV RNA 1 gene for resistance to AMV infection.

Independent putative transformed white clover plants were selected for each binary vector – pGA492RNA1 (wild type – coded as 'W'), pGA492RNA1(G) (Mutant G coded as 'G') and pGA492RNA1(T) (Mutant T coded as 'T') – and were confirmed to be transgenic by PCR analysis for the presence of the *nptII* gene. Each transgenic line had at least 10 and up to 16 clones made through tissue culture.

The untransformed control plants selected for comparison with the transformed plants are three genotypes of the white clover cultivar Haifa with a range of susceptibilities to AMV infection. The H12 genotype has relatively low susceptibility to AMV infection, followed by HNN with the HC genotype being the most susceptible. The transformed white clover plants are, as described in Section 2.4, also based on the white clover cultivar Haifa.

Replicate cuttings of a similar size and growth habit with 10 to 15 leaves were selected and inoculated at two different concentrations of AMV isolate WC28 virus inocula at the one time. The untransformed control plants 'H12', 'HC' and 'HNN' were inoculated with the 'W' and 'T' lines in one experiment and in a later experiment the same control plants were inoculated with the 'G' lines.

The results of the first inoculation are given in Table 3. The 'W' lines had similar levels of infection as the untransformed controls. Further, the symptoms of AMV

Table 3: Results of the assessment of untransformed and transformed white clover plants following inoculation with AMV isolate WC28 virus infected sap at two dilutions (1:10 and 1:5 w/v). The untransformed white clover plants are strains H12, HC and HNN. The transformed plants have been engineered to express the wild type (W series) and mutant for ATP binding (T series) AMV RNA 1 genes. Symptom severity is rated from very mild (least severe), mild, moderate severe to severe (most severe).

Plant Line	Number of plants infected/inoculated		Plants infected (%)	Symptom severity	Rating
	Virus inoculum level, 1:10	Virus inoculum level, 1:5			
H12	2/3	2/3	67	Moderate Severe	Susceptible
HC	3/3	3/3	100	Moderate Severe	Susceptible
HNN	3/3	3/3	100	Moderate Severe	Susceptible
W1	3/3/	3/3	100	Moderate Severe	Susceptible
W2	3/3	3/3	100	Moderate Severe	Susceptible
W9	3/3	3/3	100	Moderate Severe	Susceptible
W10	3/3	2/3	83	Moderate Severe	Susceptible
W20	3/3	3/3	100	Moderate Severe	Susceptible
W25	2/3	3/3	83	Severe	Susceptible
W31	2/3	2/3	67	Moderate Severe	Susceptible
W33	3/3	3/3	100	Moderate Severe	Susceptible
T2	3/3	3/3	100	Very mild	Susceptible
T6	2/3	3/3	83	Mild	Susceptible
T7	0/3	0/3	0	No symptoms	Immune
T8	1/3	3/3	67	Moderate Severe	Susceptible
T9	0/3	2/3	33	Moderate Severe	Resistant
T10	3/3	3/3	100	Moderate Severe	Susceptible

infection, clearing between the veins of the leaves and localized necrosis, in the 'W' lines were identical to that of the control plants (Figures 28 and 29). In the case of the 'T' lines, a range in the symptoms of virus infections were observed. None of the T7 lines showed any symptoms of virus infection (Figure 30). The T9 line had only two out of the three plants inoculated at the 1:5 w/v dilution showing infection by the virus with the symptoms of these plants being the same as that for the control and 'W' lines. The T6 line had 5 out of the 6 plants showing symptoms of virus infection but the symptoms were very much attenuated and described as being mild. All plants of the T2 line showed symptoms of infection but of all of the infected plants the symptoms were the most attenuated, to the point of being very difficult to identify (Figure 31).

A bioassay, involving the inoculation of cowpeas with extracted sap, for the AMV infection was carried out on all plants. From the bioassay, all plants except those of the T7 line were shown to be infected with AMV. The six inoculated plants of lines H12, HC, HNN, W9, W25, T2, T6 and T7 were grown for a further two months in the glasshouse and were analysed for total biomass production. The results, summarized in Figure 32, show that the biomass yield of the inoculated untransformed plants (H12,HC, HNN) and the 'W' series plants (W9 and W25) was less than that of the 'T' series plants. The inoculated T7 line plants had the same yield as the un-inoculated plants. The un-inoculated plants from all lines were of a similar size at the time of biomass harvest.

The results of the second experiment involving the inoculation of the untransformed control and 'G' lines are given in Table 4. The control plants H12, HC and HNN were

Figure 28: Symptoms of AMV infection on 'H12' (untransformed) white clover plants. In A) on the left is an un-inoculated plant and on the right a plant inoculated with a 1:5 dilution of AMV isolate WC28 viral inocula. In B) the lower three leaves represent those from a plant inoculated as described above whilst the upper three leaves are from an un-inoculated plant.


A)


B)

Figure 29: Symptoms of AMV infection on 'W25' ('W' line transformed) white clover plants. In A) on the left is an un-inoculated plant and on the right a plant inoculated with a 1:5 dilution of AMV isolate WC28 viral inocula. In B) the lower three leaves represent those from a plant inoculated as described above whilst the upper three leaves are from an un-inoculated plant.


A)

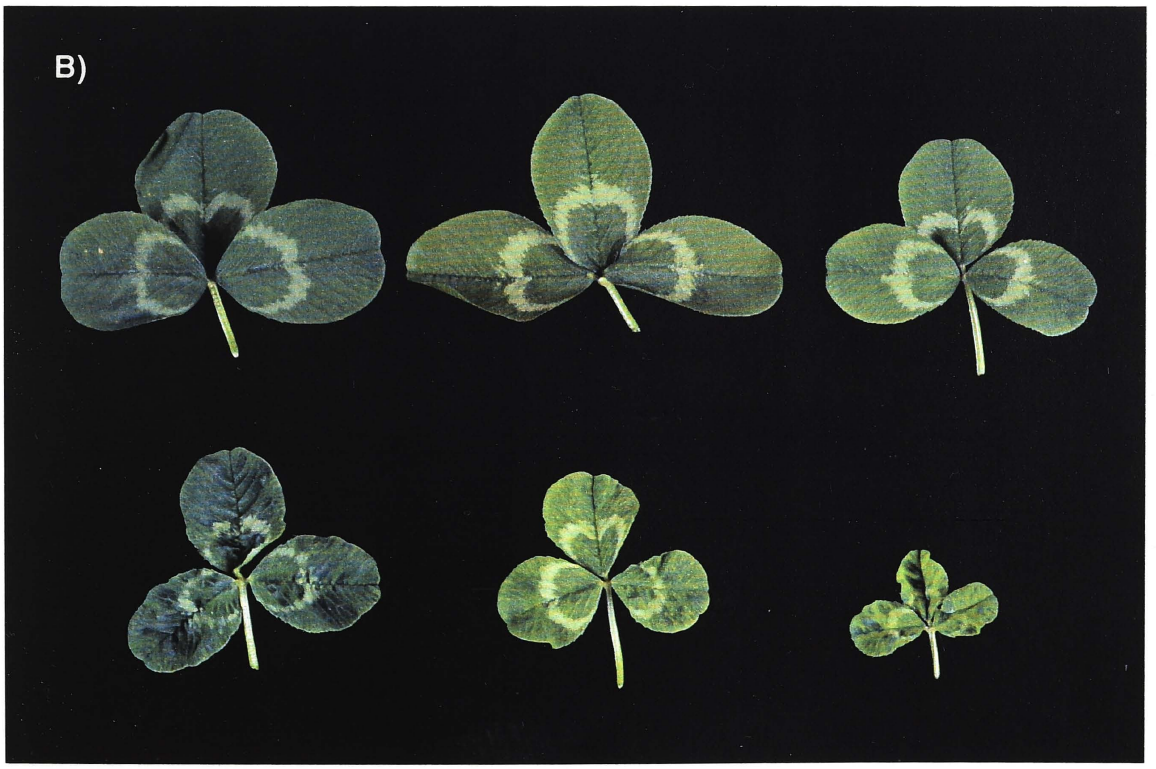

B)

Figure 30: Symptoms of AMV infection on 'T7' ('T' line transformed) white clover plants. In A) on the left is an un-inoculated plant and on the right a plant inoculated with a 1:5 dilution of AMV isolate WC28 viral inocula. In B) the lower three leaves represent those from a plant inoculated as described above whilst the upper three leaves are from an un-inoculated plant.


A)


B)

Figure 31: Symptoms of AMV infection on 'T2' ('T' line transformed) white clover plants. In A) on the left is an un-inoculated plant and on the right a plant inoculated with a 1:5 dilution of AMV isolate WC28 viral inocula. In B) the lower three leaves represent those from a plant inoculated as described above whilst the upper three leaves are from an un-inoculated plant.


A)


B)

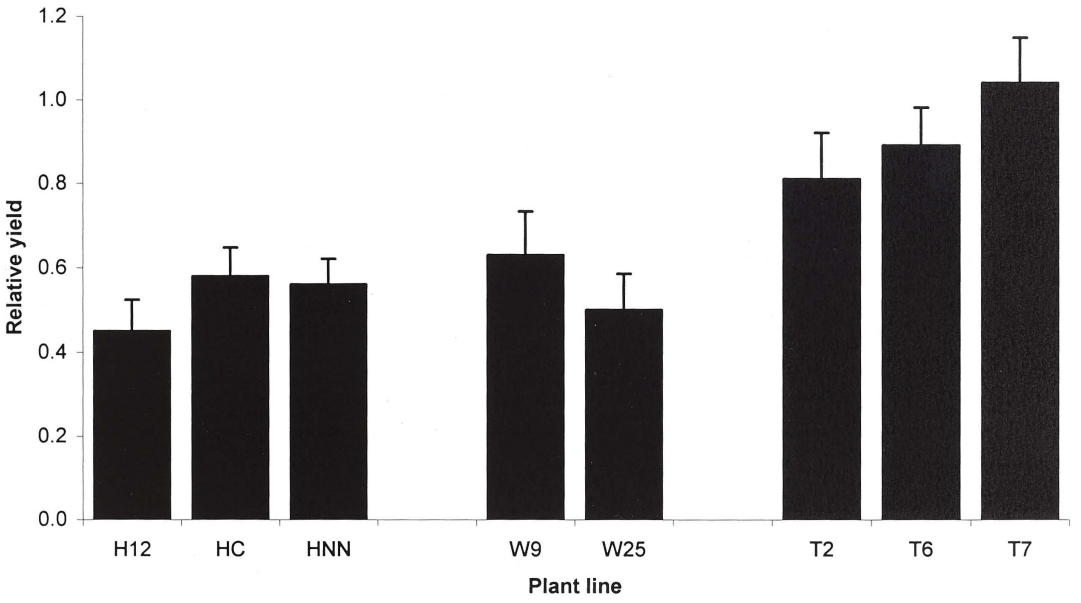


Figure 32: Relative dry weight herbage biomass yield of different white clover lines inoculated with AMV isolate WC28 compared to the un-inoculated plants of the same line. Six plants were inoculated for each line, three at 1:10 w/v dilution of the inocula and three at 1:5 w/v dilution of the inocula. Two plants were un-inoculated for each line. The mean (+s.d.) values are shown.

Table 4: Results of the assessment of untransformed and transformed white clover plants following inoculation with AMV isolate WC28 virus infected sap at two dilutions (1:10 and 1:5 w/v). The untransformed white clover plants are strains H12, HC and HNN. The transformed plants have been engineered to express the mutant for ATP binding (G series) AMV RNA 1 gene. Symptom severity is rated from very mild (least severe), mild, moderate severe to severe (most severe).

Plant Line	Number of plants infected/inoculated		Plants infected (%)	Symptom severity	Rating
	Virus inoculum level, 1:10	Virus inoculum level, 1:5			
H12	2/4	3/4	63	Severe	Susceptible
HC	4/4	4/4	100	Severe	Susceptible
HNN	4/4	3/4	88	Severe	Susceptible
G1	4/4	3/4	100	Mild	Susceptible
G2	3/3	3/3	100	Moderate severe	Susceptible
G3	3/4	4/4	88	Moderate severe	Susceptible
G4	4/4	4/4	100	Severe	Susceptible
G5	2/2	3/3	100	Moderate severe	Susceptible

similarly infected as in the previous experiment with all plants having severe symptoms of virus infection. All of the G1 plants showed symptoms of AMV infection but they were attenuated and are described as being mild (Figure 33). The symptoms of virus infection on the G2, G3 and G5 lines were a little attenuated compared with the control plants. All plants of the G4 line were infected and showed severe symptoms of virus infection, typical of that of the control lines (Figure 34).

3.3 Molecular analysis of AMV RNA 1 wild type and mutant transgene expression in transgenic tobacco and white clover.

RNA was extracted from a range of leaves for each of the tobacco and white clover control and transgenic lines. Northern blot analysis was conducted on the RNA samples to detect both the AMV RNA 1 gene mRNA and the *nptII* gene mRNA. Great difficulty was encountered in detecting the mRNA of the AMV RNA 1 gene and the mutant derivatives in both tobacco and white clover. Several different RNA extractions were tried along with probes made from the DNA of pCa17TH, the *HindIII XbaI* fragment containing DNA coding for the ATP binding motif from pCa17TH and from a 1000bp PCR amplified DNA fragment of the 5' terminal end of the coding region of the AMV RNA 1 gene.

For some tobacco plants, a band of the approximate size to that expected could be observed (4.2 kb) although this was not reproducible (Figure 35A). No band of the approximate size expected could be detected in the RNA from the white clover lines. The best northern blot achieved is shown in Figure 35 which used the RNA samples

Figure 33: Symptoms of AMV infection on 'G1' ('G' line transformed) white clover plants. In A) on the left is an un-inoculated plant and on the right a plant inoculated with a 1:5 dilution of AMV isolate WC28 viral inocula. In B) the lower three leaves represent those from a plant inoculated as described above whilst the upper three leaves are from an un-inoculated plant.


A)


B)

Figure 34: Symptoms of AMV infection on 'G4' ('G' line transformed) white clover plants. In A) on the left is an un-inoculated plant and on the right a plant inoculated with a 1:5 dilution of AMV isolate WC28 viral inocula. In B) the lower three leaves represent those from a plant inoculated as described above whilst the upper three leaves are from an un-inoculated plant.


A)


B)

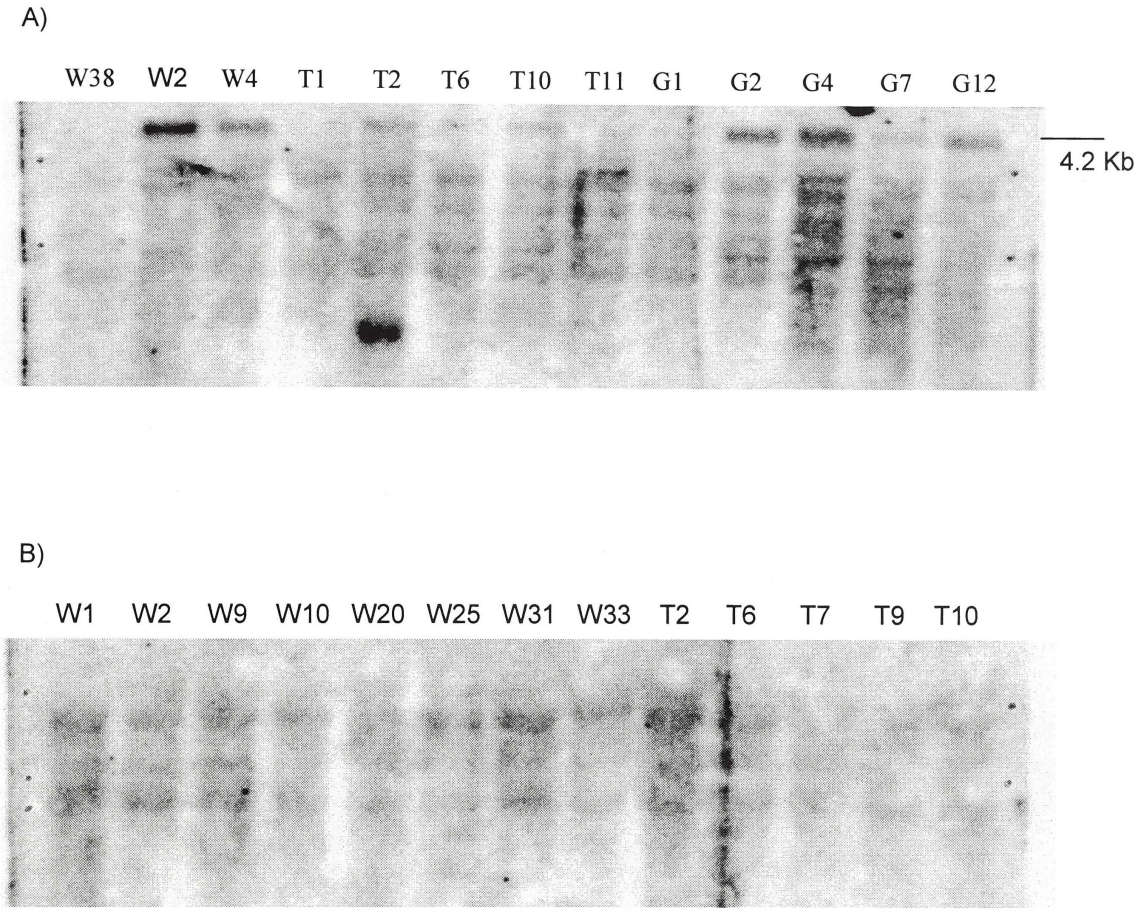


Figure 35: Northern blots using a probe derived from a fragment of the AMV RNA 1 gene in leaf RNA samples from A) tobacco; B) white clover. The film was exposed to the membrane for 6 days before developing. Bands of an approximate size to that expected were observed in some of the tobacco lines. W38 is an untransformed control.

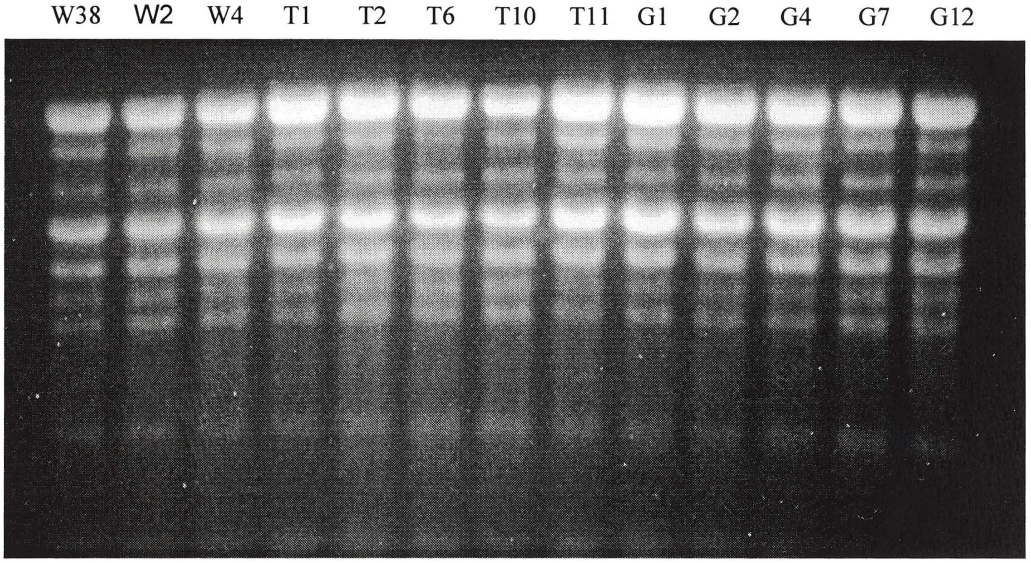
separated by agarose gel electrophoresis (Figure 36). The northern blot shown in Figure 35 is from a hybridisation using a probe that had been random primed from a *HindIII XbaI* fragment from pCa17TH containing the DNA coding for the ATP binding site and which had been exposed to film for 6 days.

Given the apparent low level of expression of the AMV RNA 1 wild type and mutant derivatives in both tobacco and white clover, RT-PCR for both this gene and the *nptII* gene was undertaken. Using DNAse 1 treated RNA samples (same as those shown in Figure 36), it was possible to detect the *nptII* gene in all samples (Figure 37). Despite many attempts using different conditions and probes, mRNA corresponding to the AMV RNA 1 gene could not be clearly detected. However in RNA samples that had not been treated with DNAse 1 the AMV RNA 1 gene was detected but not in the negative controls (Figure 38). These results indicated that the *nptII* gene is being expressed in all of the transgenic plants and although the AMV RNA 1 gene is present in the genome of the plants it is either being expressed at a low level or some form of gene silencing is taking place.

4. DISCUSSION.

In the previous section the results of inoculating transgenic tobacco and white clover plants containing AMV RNA 1 derived genes with AMV isolate WC28 viral inocula is presented. The 'W' line plants for both tobacco and white clover had a comparable level of virus infection and similar symptoms to that of the untransformed lines (in the case of tobacco 'W38' and white clover 'H12', 'HC' and 'HNN'). None of the 'W'

A)



B)

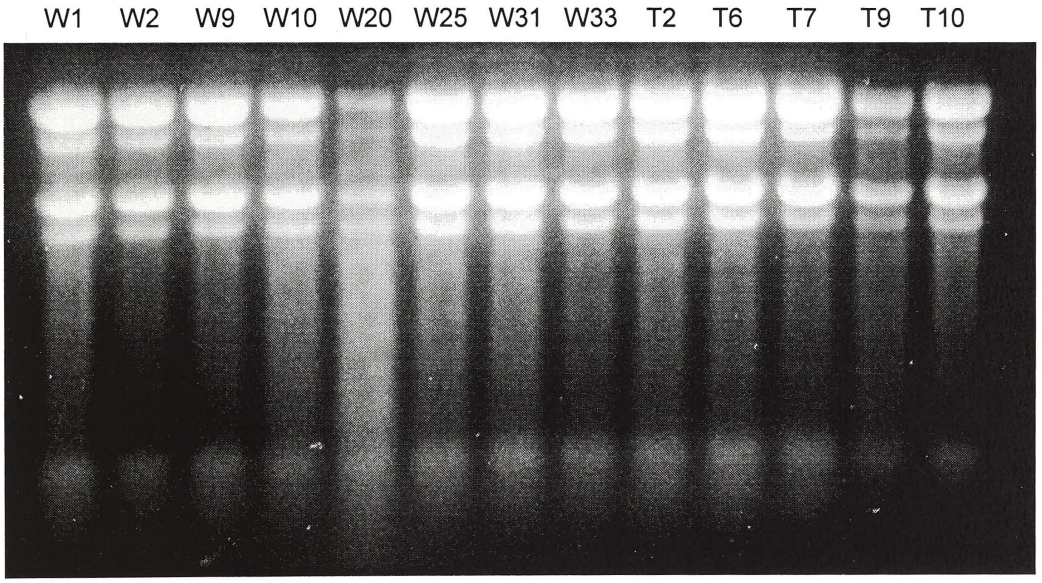


FIGURE 36: Electrophoretic analysis of RNA samples obtained from the leaves of A) tobacco and B) white clover. 10ug of RNA is loaded per lane.

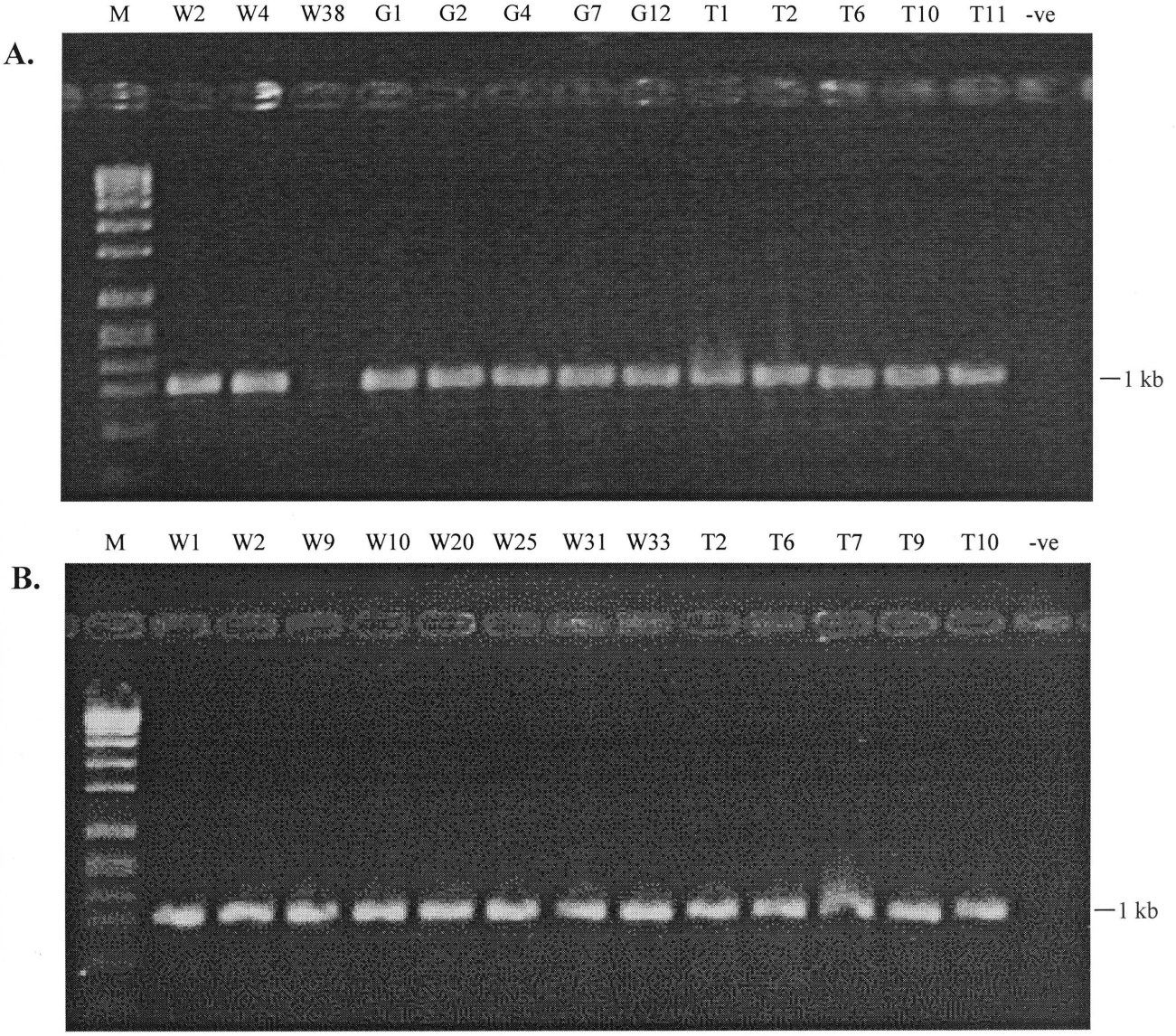


Figure 38: RT-PCR analysis of AMV transcript in RNA from A) tobacco and B) white clover. The bands observed are of the expected size. Samples '-ve' contain no template. 'W38' is an untransformed control plant. The results shown are from samples that were not treated with DNase. Samples treated with DNase failed to give any bands.

lines had symptoms of virus infection or levels of virus as determined by ELISA that was substantially different to that of the control plants. This is in contrast to the 'G' and 'T' lines in both tobacco and white clover in which a range in the severity of symptoms of virus infection and levels of virus accumulation as determined by ELISA was observed.

Most of the 'G' and 'T' lines in both tobacco and white clover had attenuated or in some cases no symptoms of virus infection. Some 'G' and 'T' lines had symptoms of virus infection and a level of virus present in representative plants similar to that of the untransformed controls and the 'W' lines in both tobacco and white clover. The results from this experiment support the hypothesis that plants expressing the 'G' and 'T' constructs produce AMV 1a protein that forms dysfunctional replication complexes with AMV 2a protein and thereby reduces or inhibits AMV infection.

Whilst the data from untransformed plants and plants containing the 'W', 'G' and 'T' constructs suggest a protein mediated form of virus resistance, with no confidence could mRNA message for the AMV RNA 1 gene could be detected in any of the transgenic plants whilst message for the *nptII* gene could be. This is an interesting result as the AMV RNA 1 gene and the *nptII* both have the 35S promoter and *nos* terminator, therefore negating any differences in gene expression on the basis of gene regulation. Furthermore, it is not conceivable that the differences (as assessed by visual score, ELISA and biomass) in virus infection in the 'G' and 'T' lines is attributable to an RNA mediated resistance mechanism as one would expect to see a similar range in virus susceptibility in the 'W' lines.

To further support the protein mediated hypothesis, no plants were seen to undergo 'recovery' and only one line (white clover T7) seemed to have high immunity to virus infection (as tested by inoculation with high levels of virus inocula). It would seem that the low level of gene expression as observed in the northern blots from the transgenic plants might be sufficient to confer resistance to virus infection and to attenuate the symptoms of infection as a protein mediated mechanism.

CHAPTER 4 SUMMARY AND GENERAL DISCUSSION.

A number of new findings are reported in this thesis. The first is the indication, by mutation of infectious clones, that the putative ATP binding motif (P-loop) in the AMV RNA 1 gene is required for virus function. This supports a previous finding that the same mutations to the P-loop in *Potato virus X* rendered the virus unable to infect plants (Davenport and Baulcombe, 1997). Further, it supports the hypothesis in conjunction with the P-loop motif being in all 1a protein sequences available for *Bromoviridae* viruses (Figure 3), of the importance of the motif to the function of the viruses in the group. Presumably this function is the binding and hydrolysis of ATP (Gorbalenya and Koonin, 1989; Saraste *et al*, 1990).

The second finding was that the insertion of 21 nucleotides into one of the conserved stem loops in the 3' untranslated region of AMV RNA 1 (loop E in Figure 14.2) reduced the infectivity of the infectious clone. This result was consistent with the previous findings that the structure of the stem loops are important for the replication and therefore the infectivity of the virus (van Rossum *et al*, 1997).

The third finding was that when plasmids based on the AMV RNA 1 infectious clone but which had a mutated ATP binding motif were co-inoculated with the unmodified AMV RNA 1 infectious clone and the other required infectious clones (RNAs 2-4), there was a lower level of infectivity of the combination than when just the unmodified infectious clone was inoculated with the RNAs 2-4 infectious clones. This suggests that the constructs with the mutated ATP binding motif produce AMV

1a protein that interferes with the infectivity and presumably the replication of the virus. I believe that the system of inoculating cowpeas with various combinations of the infectious clones is the first time that a defective plant viral protein has been shown to interfere with virus infectivity *in vivo*.

The fourth and major finding of this thesis is that plants transformed with AMV RNA 1 constructs with mutated ATP binding motifs show a range of resistance phenotypes to AMV infection whilst plants transformed with the unmodified (wild type) AMV RNA 1 construct showed no such resistance to, or attenuation of the symptoms of, virus infection. In plants transformed with the mutant forms of the AMV RNA 1 gene, the symptoms of AMV infection were substantially attenuated compared to those of untransformed plants and of plants transformed with the unmodified (wild type) gene. This is the first time that resistance to AMV infection has been reported for plants expressing modified forms of the AMV RNA 1 gene. It is also the first time that resistance to a plant virus has been reported by the expression of a replicase gene in which the ATP binding (P-loop) motif has been mutated. The resistance was shown in tobacco as a model plant system and in white clover as a commercial plant species with high susceptibility to AMV infection. The resistance was shown to be effective against AMV isolate WC28 a heterologous isolate (different to) to the source strain of the mutated transgene AMV strain 425.

The resistance to AMV infection in plants transformed with mutant forms of a gene for AMV RNA 1 is presumed to operate through a protein mediated form of resistance. Despite repeated attempts to show the presence of mRNA from the

transgenes using northern blot analysis and RT-PCR the expression of the transgene (mutant or wild type) could not be shown. This result is comparable to a recent report on transgenic tobacco expressing modified forms of the *Cucumber mosaic virus* RNA 2 gene in which translatable forms of the gene with mutations to the highly conserved GDD (RNA polymerase motif) gave a range of resistant phenotypes and attenuated symptoms of virus infection. Plants transformed with the wild type or untranslatable forms of the gene had no difference in virus susceptibility or symptoms of infection (Wintermantel and Zaitlin, 2000). They claimed their northern blot system was capable of detecting as little as 10pg of control RNA but were unable to detect the presence of the transgene mRNA in the transgenic plants. They consequently were able to show steady-state transgene message levels in the transgenic plants and the level of message positively correlated with the degree of resistance observed by using ribonuclease protection assays.

Whilst the circumstantial evidence suggests that the resistant phenotypes and attenuated symptoms of virus infection observed in plants transformed with the mutant forms of the AMV 1a protein is due to a protein mediated resistance mechanism, further evidence from molecular analysis is required to fully validate such a conclusion. Such analysis might involve a ribonuclease protection assay as discussed above or by further experimentation with the parameters of the northern blots or RT-PCR.

Alternative supporting evidence might be gained by making an AMV RNA 1 gene construct that has a silent mutation in the putative ATP binding motif – that is where

a DNA base change is made but which does not result in an altered amino acid sequence – and to evaluate plants transformed with this construct for attenuation of symptoms of infection after inoculation. Another approach might be to inoculate the tobacco and white clover plants containing the wild type gene with the complementary RNA 2-4 infectious clones and see if virus infection can take place (Bol *et al*, 1993) and argue that on the basis of the only difference between the wild type and mutant genes is a single base change in the DNA sequence coding for the ATP binding motif, that expression also takes place in those plants transformed with the mutant type.

To determine if the resistance to, and the attenuation of the symptoms of, virus infection is due to RNA mediated resistance or post transcriptional gene silencing, experiments need to be undertaken to determine if fragments of around 21 nucleotides with sequence similarity to the viral RNA (AMV RNA 1) are present in those plants which show high levels of resistance (Waterhouse *et al*, 2001).

In an attempt to gain a further understanding of degree of variability of AMV isolates in Australia, the coat protein of two different isolates in Australia were sequenced (AMV isolates H1 and YD3.2) (Appendix 1 and 2). The sequences were aligned with other published sequences and a high degree of similarity was observed (the other isolates/strains were isolated in Europe or New Zealand). On the basis of this similarity, for a related project in the research group in which I did my PhD project, I correctly predicted that if by the expression of AMV coat protein genes in plants gave rise to resistance to infection that this would be effective against a wide range

of strains and isolates. This has been the findings of such work (Paul Chu pers. comm.).

Apart from making the qualified claim that a new mechanism for resistance to virus infection in plants has been identified, I predict that the same mechanism of expressing genes that have the ATP binding motif mutated might be effective for conferring resistance to other plant viruses as well as conferring resistance to virus infection in animals and other more distantly related organisms. Given the highly conserved nature of the ATP binding motif, and that it is present in proteins with diverse functions (Gorbalenya and Koonin, 1989), mutation of the motif might be an effective way to alter metabolic pathways, regulatory signals and structural or excreted components of cells.

One of the exciting outcomes of the project is the development of a white clover line (T7) which appears to have immunity to AMV infection. Before this trait is bred into advanced breeding lines of white clover, I propose that the durability of this trait first be tested against a wide range of AMV strains/isolates and against different modes of transmission of virus, typical of those found in field conditions, especially by different aphid species.

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THE MAJOR PRACTICAL OUTCOME OF
THE PhD RESEARCH PROJECT

White clover line 'T7' – immune to AMV infection.